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THE RELEASE OF AUXIN FROM ISOLATED LEAF PROTEINS OF SPINACH BY ENZYMES

By SAM G. WILDMAN AND SOLON A. GORDON*

DEPARTMENT OF BOTANY, UNIVERSITY OF MICHIGAN†

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A number of hypotheses have been advanced concerning the substance from which auxin is liberated in plant tissues. Kögl, Erxleben and Haagen Smit[9] postulated the existence of auxin esters in fats and oils, since they were able to hydrolyze auxin from oil of *Arachis* and other plants by means of lipase or saponification with alcoholic sodium hydroxide. Recently there appeared a brief note by Skoog and Thimann[13] to the effect that an increase of auxin could be obtained from *Lemna* tissue by the addition of proteolytic enzymes. They considered that the auxin was bound to proteins. Avery, Berger and Shalucha[1] have criticized this hypothesis as being premature since proteolytic enzymes also hydrolyze simple peptides and amides. In addition, they were unable to hydrolyze the auxin precursor in a corn endosperm extract with commercial preparations of trypsin, pepsin, papain, ficin, taka-diastase, malt diastase or steapsin at pH values of 7 or less although the precursor could be hydrolyzed by other means. They suggest that the precursor in maize may be an acid amide of indole acetic acid. It is at once evident that the information concerning this phase of plant hormone research is extremely meager.

Methods for the isolation of proteins from leaves have been proposed by Chibnall,[3] Menke[11] and Granick,[5, 6] and it should be possible to test the hypothesis that auxin is associated with protein by the use of isolated plant proteins. If it can be shown that auxin is definitely a part of protein molecules or associated with them, some ramifications of the problem may be studied such as (1) whether auxin is confined to cytoplasmic or chloroplastic protein, or found in both; (2) if it is found in leaf proteins, can these proteins be further fractionated so that the yield of auxin from one fraction is greater than from another; (3) diffusion rates of the auxin released from proteins can be made to determine whether they compare with the values of auxin directly extracted from the leaf. With these considerations in mind, the following experiments were performed.

Preparation of Proteins.—Cytoplasmic proteins: Chibnall[8] has shown that vacuolar materials, and the soluble substances of the cytoplasm could be removed by cytolysis of the leaf tissue followed by pressure to "squeeze" the cell fluids from the leaf, cytoplasmic protein and chloroplasts remaining within the cell walls. The cells were then broken by grinding, the ground material suspended in water and the cell wall fragments removed by passing the liquid through silk. The chloroplasts were separated from the cytoplasmic proteins by passing the suspension through paper pulp. Menke,[11] on the contrary, ground spinach leaves without previous treatment and separated the chloroplastic protein from the cytoplasmic protein by precipitation with 25 per cent ammonium sulphate.

A combination and modification of the methods proposed by Chibnall and Menke have been used to prepare the leaf proteins of spinach for this study. Approximately one kilogram of market spinach (consisting only of leaves) was thoroughly washed and cytolyzed for a few minutes with ether. The flaccid leaf material was surrounded by cotton cheesecloth and the juice squeezed out in a hydraulic press. Upon releasing the pressure, the leaves were allowed to imbibe water and then pressed again. Imbibition and pressing were repeated three times. After the final pressure treatment, the material was again allowed to imbibe water and the leaves were passed twice through the coarse burr of a "Nixtamal" mill, then three times more using the finest burr. Following this preliminary grinding, water was added in small quantities and the leaf material was repeatedly passed through the mill until a semi-liquid consistency was obtained. The finely divided material was then dispersed in about two liters of distilled water. Cell wall debris was removed by a short centrifugation at low speed and the supernatant liquid was passed through fine mesh silk to remove all visible traces of cell fragments. The combined centrifugates were again ground and dispersed as before and both dispersions combined to give a total of about three liters of liquid.

The chloroplasts were precipitated by adding ammonium sulphate to give a final concentration of 7.5 per cent. The material was allowed to flocculate for 12 hours at 5°C., and the precipitated chloroplasts removed by filtering through Whatman No. 12 folded filter paper. The filtrate was returned through the same paper until the filtrate became free from green coloration. All filtering was done at 5°C. with toluol present to prevent bacterial decomposition of the proteins. We have used 25, 20, 15, 10 and 5 per cent ammonium sulphate to precipitate the chloroplasts in other preparations. Table 1 expresses the nitrogen (and protein) content of some of these preparations. Nitrogen was determined by the micro-Kjehldahl method using selenium as a catalyst. However, smaller yields of cytoplasmic protein than anticipated seemed to indicate that some cytoplasmic protein precipitated with the chloroplasts when high concentrations of

ammonium sulphate were used; hence, the values reported here are confined to those preparations in which not more than 15 per cent ammonium sulphate was used.

TABLE 1

NITROGEN CONTENT OF PROTEINS USED FOR AUXIN ANALYSIS. PROTEINS WERE SUBJECTED TO PROLONGED EXTRACTION WITH ETHER AND ETHER-ALCOHOL BEFORE ANALYSIS (SEE TEXT)

KIND OF PROTEIN	% $(NH_4)_2SO_4$ USED TO PRECIPITATE CHLOROPLASTIC PROTEINS	% N	% PROTEIN (N × 6.25)
Cytoplasmic, precipitated at pH 4.0	15.0	15.6	97.5
	15.0	15.5	96.9
Cytoplasmic, precipitated at pH 4.0	10.0	15.7	98.1
	10.0	15.6	97.5
Cytoplasmic, precipitated at pH 5.2	7.5	15.4	96.3
	7.5	15.5	96.9
Cytoplasmic, precipitated at pH 3.5	7.5	15.5	96.9
Cytoplasmic, precipitated at pH 5.2	5.0	15.2	95.5
	5.0	15.2	95.5
Chloroplastic, prepared by Granick's method	..	11.1	69.4
	..	10.9	68.3

Cytoplasmic protein contained in the filtrate was precipitated by adjusting the pH of the solution to 4.0 with 0.1 *N* hydrochloric acid. All pH measurements were made with a Beckmann glass electrode. The precipitate was allowed to flocculate for several hours and then collected on Whatman No. 1 filter paper. The protein precipitate was washed until free from sulphate with distilled water whose pH had been adjusted to 4.0. The precipitate was then removed from the filter paper and dried in vacuum at 37°C. The dried proteins were ground to pass through a 100-mesh screen and extracted with ether for 72 hours in a Soxhlet apparatus. This extraction was followed by a 1:4 ether-alcohol extraction for 72 hours. Both the ether and ether-alcohol extracts gave negative tests for auxin by the *Avena* assay method. A yield of four grams of cytoplasmic protein has been obtained from one kilogram of leaf tissue when the chloroplasts were precipitated with 7.5 per cent ammonium sulphate.

Chloroplastic proteins: With the object of obtaining a relatively pure sample of chloroplasts, a modification of the differential-centrifugation method suggested by Granick[5, 6] was used. Seventy-five grams of market spinach were macerated to yield a suspension in glucose solution. Centrifuging seven minutes at 700 RCF threw out the heavy cell debris, crystals, starch granules and clumps of chloroplasts. The supernatant liquid was decanted and centrifuged ten minutes at 1150 RCF to yield a centrifugate with a white layer, probably suspended sand and porcelain, at its lower portion and covered by a layer of chloroplasts. The supernatant liquid of this

deposit was centrifuged fifteen minutes at 1490 RCF to yield a centrifugate of dark green color. Microscopic examination of this showed mostly isolated chloroplasts with an occasional fragment. To remove the glucose, the chloroplasts were suspended and centrifuged twice in distilled water fifteen minutes at 1490 RCF. Then, they were dried *in vacuo* at 37°C., ground to 100 mesh and extracted successively with ether and ether-alcohol. The nitrogen content of this preparation is given in table 1.

Digestions of Proteins by Enzymes.—All digestions of protein herein reported have been carried out under constant conditions. Preliminary experiments indicated that the maximum rate of digestion with tryptic enzymes occurred between a pH of 7.0 and 9.0, using a KH_2PO_4-NaOH buffer medium. It was likewise found that 72 hours was the minimum time that could be used to digest the proteins and still obtain consistent yields of auxin. Inasmuch as proteolytic enzymes of animal origin appear to attain their maximum efficiency at temperatures near that of blood,[4] an incubation temperature of 37°C. was used. Digestions were made with 10 mg. of protein suspended in 10 ml. of buffer solution at pH 8.0 with the exception of those digestions employing papain in which 10 ml. of phosphate buffer at pH 4.5 and 6.0 were used. Four to five drops of toluol were added to each digestion flask as an antiseptic and renewed during the digestion period if necessary; each flask was tightly stoppered to prevent evaporation of the toluol. At the completion of the 72-hour digestion period, the pH of the solution was adjusted to 4.0–4.5, the solution transferred to a separatory funnel and shaken twice with 75 to 100-ml. portions of recently distilled ether. Ether containing the auxin was separated from the protein solution and evaporated at low temperature until only a few milliliters remained. These were transferred to a small vial to which agar was added.

Auxin Determinations.—Auxin was determined by the standard *Avena* test procedure,[16] using a reaction time of 90 minutes and a uniform intermediate decapitation time of 2–2.5 hours. All values herein reported are based on determinations made within the dilution range. Dilutions were made either by diluting the agar which contained the hormone, or by dissolving the extract of the hormone in ether, making up to volume with ether at 5°C., and removing suitable aliquots. Although van Overbeek's[12] formula is of utility in *Avena* determinations, the constants involved when 10 to 40-mg. samples are used are of such magnitudes as to make its use impractical in expressing the results obtained here. Hence, the values expressed are actual *Avena* curvatures per 0.35 ml. of agar and comparisons are based upon determinations made on the same day to obviate correcting for differences in sensitivity of the test plants.

Enzymes Used.—We have repeatedly tested various concentrations of the enzymes used for the presence of auxin; the *Avena* assay was always nega-

tive. They were tested before and after 72 hours' incubation under conditions identical with the conditions of protein digestions.

Trypsin and chymotrypsin were commercially prepared crystalline enzymes. Papain‡ was a twice crystallized preparation dissolved in 0.2M NaCN and had an activity of 10 milk-clotting units per 3.7 mg. of protein. What is called tryptic extract in this paper is a highly purified, non-crystalline trypsin commercially prepared from pancreas and presumably containing a mixture of proteolytic enzymes.[14] Preliminary experiments disclosed no significant difference in auxin yield when 0.5, 1.0 and 5.0 mg. of crystalline enzymes per 10 mg. of protein were used; therefore, 1.0 mg. of crystalline enzyme was used for each digestion with trypsin or chymotrypsin. Similarly, 1.0, 5.0 and 10.0 mg. of tryptic extract were not markedly different in ability to yield auxin. Accordingly, 5.0 mg. of enzyme was added to each digestion in which tryptic extract was used. 1.0 mg. of papain was used in those digestions entailing this enzyme.

Bacterial Contamination.—The possibility exists that auxin liberated from protein may be due to the action of bacteria, either on the protein or on the hydrolytic products of the protein. However, we feel that the following considerations will substantiate our later thesis that the auxin is released by enzymatic hydrolysis and not through the functioning of microörganisms: (1) Toluol was employed as an antiseptic. Any digestion in which the odor of toluol could not be clearly detected at the time of neutralization was discarded. (2) Microscopic examination of the clear liquid from digestions to which toluol had been added disclosed no microörganisms; when toluol was omitted, the liquid became cloudy and its odor offensive; microscopic inspection revealed numerous organisms. (3) No turbidity or odor had developed in a tightly stoppered flask containing 40 mg. of protein, 40 ml. of buffer, 4.0 mg. of chymotrypsin and four drops of toluol at the end of three months. (4) The yields of auxin obtained from like protein and enzyme in various experiments were markedly uniform. That this reproducibility should exist with fortuitous contamination of the digestions by air-borne spores is not probable. (5) Twelve protein digestions were prepared as previously described with trypsin, chymotrypsin and tryptic extract in the presence of toluol. After 72 hours' incubation, 1 ml. of each was plated out upon beef-peptone agar using sterile transfer technique. A few drops of toluol were then added to eight of the plates, none being added to the remaining four. No bacterial or fungus colonies developed within ten days in those plates protected with toluol. Conversely, numerous colonies developed within four days on the four plates not protected by toluol. Undoubtedly the digestions do contain a few microorganisms, but we are convinced that toluol keeps them inactive.

Experimental Results: Hydrolysis of Proteins to Yield Auxin.—Examination of the data presented in table 2 clearly demonstrates that auxin is

associated with protein, and is released through the action of proteolytic enzymes. Like results have also been obtained for three other protein preparations, and always there has been complete corroboration of the data presented. Since the dry protein was suspended in buffer solution, liquefaction of the protein and attainment of a clear solution is assumed to represent hydrolysis. Protein controls, to which no enzyme had been added, failed to dissolve completely during a period of 72 hours, although there sometimes appeared to be a small amount of hydrolysis as attested by *Avena* curvatures. Chibnall has shown that there is a gradual auto-digestion when cytoplasmic proteins are maintained in aqueous solution or in a highly hydrated form. The inference is that intracellular enzymes are present and accomplish this hydrolysis. Thus, one would expect a small amount of auxin to be normally produced in the absence of added enzyme.

TABLE 2

AUXIN RELEASED FROM TWO DIFFERENT PREPARATIONS OF CYTOPLASMIC PROTEINS. DUPLICATES ARE THE RESULT OF TWO SEPARATE *Avena*-CURVATURE DETERMINATIONS WITH TWO SEPARATE PROTEIN DIGESTIONS

PROTEIN PREPARATION	NO. OF 10-MG. DIGESTIONS	ENZYME	AVERAGE *Avena* CURVATURE IN DEGREES
Cytoplasmic proteins precipitated at pH 4.0, after precipitation of chloroplastic proteins by 10% $(NH_4)_2SO_4$	4*	Control	0
			2.0 ± 0.3
	4	Chymotrypsin	4.4 ± 0.4
			3.8 ± 0.5
	4	Trypsin	6.0 ± 0.4
			4.8 ± 0.6
	4	Tryptic extract	15.0 ± 0.6
			12.5 ± 0.7
Cytoplasmic proteins precipitated at pH 4.0, after precipitation of chloroplastic proteins by 15% $(NH_4)_2SO_4$	4	Control	0
			0
	4	Chymotrypsin	2.0 ± 0.9
	4	Trypsin	0
	4	Tryptic extract	8.4 ± 0.8
			9.1 ± 0.4
Cytoplasmic proteins precipitated at pH 5.2, after precipitation of chloroplastic proteins by 7.5% $(NH_4)_2SO_4$	3	Control	0
	4	Control	0
			1.3 ± 0.5
	4	Trypsin	0
	2	Chymotrypsin	0
	4	Chymotrypsin	0
	1	Tryptic extract	1.0 ± 0.3
			2.6 ± 0.8
	3	Tryptic extract	2.9 ± 0.6
	4	Tryptic extract	8.5 ± 1.3

* Ether extracts of four separate digestions combined.

When the three enzymes are compared as to their efficacy in causing auxin to be released from protein, tryptic extract (so far as our data go) is the best. Trypsin and chymotrypsin are less effective. Little, if any, visible hydrolysis of proteins resulted when papain was used either at pH 4.0 or 6.0. The results were inconclusive. For instance, one experiment would show an apparently significant increase in auxin but an attempt to duplicate it would fail. The amount of papain available was insufficient to determine the optimum conditions for the hydrolysis of leaf proteins. The most significant fact here, however, is the reproducibility of the results: two separate digestions of equal weight of protein yield nearly equal amounts of auxin as measured by *Avena* curvatures.

Isoelectric Fractionation of Cytoplasmic Proteins.—After the chloroplasts had been removed by precipitation with ammonium sulphate and filtering, 0.1 *N* hydrochloric acid was added to a filtrate containing cytoplasmic proteins until a pH of 5.2 was attained. At this hydrogen-ion concentration, a rapid and voluminous flocculation of protein occurred. Flocculation

TABLE 3

AUXIN YIELD FROM TWO FRACTIONS OF CYTOPLASMIC PROTEINS OBTAINED BY ISOELECTRIC PRECIPITATION. (CHLOROPLASTIC PROTEIN HAD BEEN SEPARATED FROM CYTOPLASMIC PROTEIN BY 7.5% $(NH_4)_2SO_4$)

NO. OF 10-MG. DIGESTIONS	ENZYME	AVERAGE *Avena* CURVATURE IN DEGREES PROTEIN PREPARATION PRECIPITATED AT pH 5.2	PRECIPITATED AT pH 3.5
4	Control	1.3 ± 0.5	5.2 ± 0.7
1	Control	0	0
1	Chymotrypsin	0	9.8 ± 1.2
2	Chymotrypsin	0	16.8 ± 1.0
1	Trypsin	0	6.2 ± 0.7
		0	1.4 ± 0.2
1	Tryptic extract	1.0 ± 0.3	15.3 ± 1.1
		1.3 ± 0.4	

was allowed to proceed at room temperature for one-half hour, and the precipitate was then removed by filtration at 5°C. which required about six hours. The filtrate was then adjusted to pH 3.5 which precipitated more protein. This was removed by filtration. Both precipitates were thoroughly washed with water properly acidified, dried *in vacuo* at 37°C. and extracted with ether and ether-alcohol. The protein fraction precipitated at pH 3.5 constituted about five per cent of the total cytoplasmic protein. Auxin yields obtained by enzymatic hydrolysis of these two cytoplasmic protein fractions appear in table 3.

When considered on a basis of protein weight, it will be noticed that the protein fraction precipitated at pH 3.5 contains considerably more auxin than the protein precipitated at pH 5.2. Here again, the same trend is evident: tryptic extract releases more auxin than trypsin or chymotrypsin.

The presence of intracellular enzymes capable of hydrolyzing proteins and releasing auxin is suggested by the relatively large curvature obtained in the control without enzymes using four 10 mg. digestions of protein. However, the control consisting of one 10 mg. digestion of protein should be used for comparison with those digestions to which enzymes were added.

Auxin Yield from Chloroplastic Proteins.—The values in table 4 disclose that auxin is also obtained by enzymatic hydrolysis from a protein preparation of spinach chloroplasts. From table 1, it is obvious that the chloroplastic protein preparation is of lower purity than the cytoplasmic protein preparations. If the curvatures in table 4 are weighted on a per-milligram-of-nitrogen basis, a better comparison can be obtained between the auxin content of the two proteins. Of course, this is made on the assumption that auxin is associated only with the protein of the chloroplasts and cytoplasm. The values for auxin obtained from chloroplastic protein were increased by 40 per cent; table 5 shows these curvatures weighted to allow for impurities. Compensating for the impurity in the chloroplastic protein, the two proteins yielded approximately the same amount of auxin with trypsin and tryptic extract, but not with chymotrypsin.

TABLE 4

AUXIN YIELD FROM CHLOROPLASTIC AND CYTOPLASMIC PROTEINS

NO. OF 10-MG. DIGESTIONS	ENZYME	AVERAGE *Avena* CURVATURE IN DEGREES CHLOROPLASTIC PROTEIN (BY GRANICK'S METHOD)	CYTOPLASMIC PROTEIN PRECIPITATED AT pH 3.5
2	Control	2.6 ± 0.6	3.5 ± 0.4
1	Control	0	0
1	Trypsin	3.9 ± 0.5	6.2 ± 0.7
2	Chymotrypsin	4.5 ± 0.9	16.8 ± 1.0
1	Chymotrypsin	0.5 ± 0.2	9.8 ± 1.2
1	Tryptic extract	15.8 ± 1.0	15.3 ± 1.1
		11.6 ± 0.8	

TABLE 5

AUXIN YIELD FROM CHLOROPLASTIC AND CYTOPLASMIC PROTEINS COMPARED ON A PER-MILLIGRAM-OF-NITROGEN BASIS

NO. OF 10-MG. DIGESTIONS	ENZYME	AVERAGE *Avena* CURVATURE IN DEGREES CHLOROPLASTIC PROTEIN	CYTOPLASMIC PROTEIN
2	Control	3.6	3.5
1	Control	0	0
1	Trypsin	5.5	6.2
2	Chymotrypsin	6.3	16.8
1	Chymotrypsin	0.7	9.8
1	Tryptic extract	19.2*	15.3

* Average of two determinations.

Diffusion Experiments.—The question can be raised whether the auxin released from the proteins is similar to the auxin found in the leaf. Corresponding diffusion rates of the two auxins should indicate a similarity in

molecular weights. The diffusion technique has been described by a number of workers;[8, 15] accordingly, experiments were performed in general following previous procedures comparing the auxin obtained from ether extracts of spinach leaves to the auxin liberated by enzymatic hydrolysis of the cytoplasmic protein. At the same time, these auxins were compared with a growth substance of known constitution, crystalline indole acetic acid. Agar blocks containing the auxin preparations, purified by

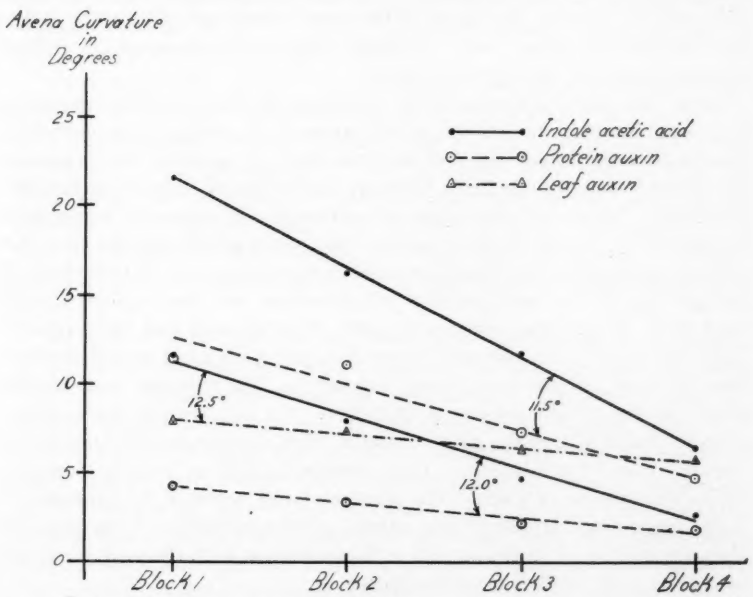

FIGURE 1

The distribution of auxin in four agar blocks, each 1.6 mm. thick, after 90 minutes' diffusion. The two upper curves represent one experiment, while the three lower curves are from another experiment. The initial concentration of indole acetic acid was 100 gammas per liter.

the donor-block method suggested by van Overbeek,[12] were placed upon three blocks of pure agar, each 1.6 mm. thick, and diffusion was allowed to ensue for 90 minutes.

If the concentrations of auxin in the four blocks are within the proportionality range, the resulting *Avena* curvatures plot graphically as a straight line. The slope of this line is dependent upon the time of diffusion, the temperature and the weight of the auxin molecule. If the temperature and time of diffusion are held constant for the auxins under comparison in a

given experiment, the slopes should vary as a function of the molecular weights. Hence, we have used contrasting slopes as a criterion of dissimilar molecular weights.

In figure 1 are shown data from two diffusion experiments. The angular difference between the indole acetic acid and enzyme auxin curves of one experiment is 11.5°; in the other experiment, it is 12.0°, and the angular difference between the leaf auxin and indole acetic acid curves is 12.5° Parallel data have been obtained from repeated experiments. There is a dissimilarity between the slopes of the curves obtained with indole acetic acid and enzyme auxin, and a similarity between the slopes of the curves obtained with enzyme and leaf auxins.

Discussion and Interpretation of Experimental Results.—The preceding data establish that an *Avena* growth-accelerating substance is definitely associated with proteins isolated from the leaves of spinach; the auxin can be released from the proteins through the action of various proteolytic enzymes. Tryptic extract appears to yield a greater amount of auxin than crystalline trypsin or chymotrypsin. This result would indicate that the absolute amount of auxin released depends upon the extent of hydrolysis of the proteins, if it is assumed that tryptic extract contains a mixture of at least three proteolytic enzymes, trypsin, chymotrypsin and carboxypolypeptidase[2] and hence, will split a protein more completely than the crystalline enzymes. However, it is not argued that the enzymes used in this study represent those present in the plant. On the contrary, we consider it more plausible that the plant contains other enzymes which function to release auxin from proteins. These results explain at least in part the hydrolytic release of auxin from plant material reported by Gustafson,[7] Link, Eggers and Moulton[10] and others. The elucidation of the position that auxin occupies in the protein or in association with the proteins must, of course, await further experimentation.

The possibility that association of auxin with proteins is an adsorption phenomenon should be considered. We feel that the evidence weighs against a hypothesis of purely physical adsorption. In the first place, auxin released from proteins by hydrolysis is organophilic; hence, if it were adsorbed on proteins, prolonged extraction with organic solvents should elute the auxin from the protein. No auxin has been obtained by extraction of the proteins with ether and ether-alcohol. Second, liquefaction of proteins by enzymes is complete within twelve hours, but auxin cannot be obtained consistently until the proteins have been hydrolyzed for 72 hours. If the process is one of adsorption, it might reasonably be assumed that auxin would be released as soon as the protein had been liquefied.

It seems unlikely that substances of low molecular weight such as amides, peptides, etc., should be contained in these protein preparations. The washings after cytolysis of the leaf to remove cell fluids, together with

the narrow pH range of cytoplasmic protein precipitation, and extensive washing of the precipitates throughout the process of preparation would tend to remove water soluble impurities.

We hold that our experiments are valid for spinach proteins and are verifiable on the same material. Although one might extend these results to other leaf material on the basis of Chibnall's work which showed that the differences were "strikingly small" between the leaf proteins prepared from five separate plant families, one would certainly not be justified without extensive further work in drawing an analogy between these proteins and those contained in seeds, fruits, etc.

The consideration that auxin is found in both the chloroplastic and cytoplasmic proteins leads to an interesting speculation: does auxin function in the synthesis of these proteins, or is auxin represented as an end-product of protein metabolism? If the former hypothesis can be substantiated, then the presence of auxin in both fractions can perhaps be explained. If the latter is found to be the true explanation, why is auxin not confined to the cytoplasm which is in contact with the cell wall upon which its function has been explained, according to Went and Thimann, by Heyn?

It has been shown that the auxin content of one fraction of cytoplasmic protein is greater than in another when the two fractions have been separated from each other by isoelectric precipitation. This fact at once suggests the desirability of separating and crystallizing these proteins. Not until this has been accomplished can an adequate study of the amount of auxin released in relation to the extent of protein hydrolysis be undertaken, since at the present stage it is impossible to tell if auxin is associated with some proteins, but not with others, or associated with all proteins but in varying amounts. Chibnall believes that protein preparations of leaves "are really mixtures of many proteins which have similar solubility properties" (p. 141), although they can be conveniently classified as glutelins on the basis of their insolubility in water and solubility in a slight excess of either acid or base. It may also be possible to isolate the enzymes responsible for auxin release in the plant since they are apparently present in some of the protein preparations.

The identification of the auxin released by enzymatic hydrolysis of the leaf protein with the free auxin of the leaf can only be determined by actual isolation of the auxins. From the data presented in figure 1, and other experiments, it appears that the auxin released from proteins is of similar molecular size to the free auxin of the leaf. The dissimilarity between the slopes of the curves for enzyme and leaf auxin, on the one hand, as contrasted with those for indole acetic acid on the other suggests that the auxin in the leaf and the auxin released from proteins are of a lower molecular weight than indole acetic acid.

Summary.—1. Auxin has been shown to be associated with proteins isolated from the leaves of spinach. It can be released by enzymatic hydrolysis.

2. Auxin is obtained from both the cytoplasmic and chloroplastic proteins by enzymatic hydrolysis of the proteins. The cytoplasmic proteins have been separated into two fractions by isoelectric precipitation; more auxin is obtained from one of these fractions than from the other.

3. Diffusion experiments indicate the similarity between the leaf auxin and the auxin released from proteins by enzymatic hydrolysis. Both auxins appear to be of lower molecular weight than indole acetic acid.

4. Evidence is presented that the auxin released from leaf proteins of spinach is not a result of bacterial contamination.

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THE DISTRIBUTION OF X-RAY INDUCED CHROMOSOMAL ABERRATIONS

By KARL SAX

ARNOLD ARBORETUM, HARVARD UNIVERSITY*

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The distribution of chromosomal aberrations in *Tradescantia* microspores is not at random for the various loci of the chromosome arms. Both spontaneous (Giles[1]) and x-ray induced (Sax and Mather[2]) aberrations occur more frequently near the centromere than in the distal loci of the chromosome arms. This distribution is found for both simple terminal deletions and for the more complex aberrations involving fusions or exchanges between different chromosomes. An excess of x-ray induced breaks in the proximal regions of the chromosome arms have been found in *Crepis* by Lewitsky and Sizova,[3] and in the grasshopper *Circotettix* by Helwig.[4] The distribution of breaks in *Drosophila* chromosomes is more nearly at random (Kaufmann[5]), but here the breaks produced by raying the sperm do not result in new associations until fertilization of the egg several days later. Many types of aberrations are eliminated, due to their lethal effect, before the salivary gland chromosomes are analyzed.

The actual or potential breaks produced in the chromosomes by x-rays must be at random, but the production of chromosomal aberrations resulting from deletions or from illegitimate fusions between broken ends of chromosomes is determined, to a considerable extent, by secondary factors. One of these factors is the spatial relations of the chromosomes. One break in a single chromosome usually is followed by restitution; but, if the chromosome is split into two sister chromatids, a break may be followed by lateral fusion of broken ends of the two chromatids to produce a dicentric chromosome and a deletion. Fusions between broken ends of different chromosomes occur only when the breaks are in close proximity. The absence of free recombinations is shown by the proportions of the various types of chromosomal aberrations. In the salivary gland chromosomes of *Drosophila* the induced inversions, in relation to reciprocal translocations, are twice as frequent as expected.[5] In *Tradescantia* the proportion of ring chromosomes, as compared with dicentrics, is about three times that expected with free recombination of broken ends of chromosomes. It is evident that propinquity favors aberrations within a single chromosome.

Another factor in the production of effective breaks in chromosomes is the rôle of the centromere in relation to the coiling cycle and polarity of the chromosomes. Acentric chromosome fragments are less sensitive to x-rays than are the normal chromosomes.[6] When breaks occur in adjacent

arms of different chromosomes the illegitimate fusion may result in either a translocation or a dicentric chromosome. The analysis of such aberrations induced at prophase shows that dicentrics are twice as frequent as translocations.[7] This non-random distribution indicates that lateral fusions are more frequent than diagonal fusions in the polarized chromosomes, and suggests that the coiling mechanism of the chromosomes at this stage of nuclear development may impose stresses which separate the chromosome at the point of breakage. The analyses of differential sensitivity of chromosomes to x-rays[8] also support the assumption that chromosome movement is a potent secondary factor in the production of chromosomal aberrations. Chromosome orientation and chromosome movement are, to a considerable extent, under the control of the centromere.

The relative sensitivity of centric and acentric chromosomes to x-ray has been determined by analyzing the results of successive exposures. The microspores were rayed during the resting stage to produce chromosome rings and dicentrics and their accompanying acentric fragments. The same microspores were irradiated two days later at prophase, and the cells were fixed and stained a day later. The second exposure produced only chromatid aberrations which could be distinguished from the chromosome aberrations induced by the first exposure. Of the 11,502 chromosomes examined there were 681 acentric chromosome segments released by the production of ring and dicentric chromosomes. These acentric fragments were equivalent in length to 340 normal chromosomes or 3.0 per cent of the total chromosome complement. Of the 1215 chromatid breaks only 4 or 0.3 per cent were found in acentric chromosome fragments. Thus the breaks in acentric chromosomes were only about a tenth as frequent as expected if aberrations were produced at random.

The low incidence of x-ray induced aberrations in acentric chromosome fragments may be related to the absence of a centromere, or it may be associated with the low incidence of multiple aberrations in a single chromosome, either centric or acentric. The second possibility was tested by analyzing the relative frequency of the various types of aberrations induced at early prophase. The *Tradescantia* microspores were given 320 r and the cells were fixed 25–30 hours after raying. Among the 3840 chromosomes examined there were 715 chromosomes with a 1-hit chromatid aberration and 266 with a 2-hit chromatid aberration, a total of 25.6 per cent. There were also 502 chromosomes with 2-hit chromosome aberrations, or 13.8 per cent. Only 8 chromosomes were found with both a chromatid and a chromosome aberration in the same chromosome. If aberrations were at random we should expect 3.5 per cent of the chromosomes to have such multiple aberrations, but only 0.2 per cent were observed. The paucity of such multiple aberrations suggests that one effective break in a chromosome inhibits the occurrence of a second effec-

tive break, but since we are dealing with combinations of chromatid and chromosome aberrations a short period of transition from one type to the other would also account for the low frequency of these multiple aberrations.

TABLE 1

FREQUENCY OF CHROMOSOMAL ABERRATIONS. TOTAL CHROMOSOMES 2334

TYPE OF ABERRATION	PER CENT OF CHROMOSOMES OBSERVED		EXPECTED	
One 1-hit chromatid	11.8 %			
One 2-hit chromatid	15.0 %			
One 2-hit chromosome	9.4 %			
Multiple aberrations:				
Two 1-hit tid	0.04%		1.4%	
Two 2-hit tid	0.7 %		2.2%	
Two 2-hit chromosome	0.0 %	1.04	0.9%	6.3
1-hit + 2-hit tid	0.3 %		1.8%	
1-hit tid + 2-hit chromosome	0.04%	0.24	1.1%	2.5
2-hit tid + 2-hit chromosome	0.2 %		1.4%	
Total multiple aberrations	1.28%		8.8%	

A second analysis of chromatid and chromosome aberrations was made in which all of the various types of multiple aberrations were included. The cells were fixed at 30–32 hours after irradiation of the microspores. The results of the analysis are shown in table 1. The expected frequency of multiple aberrations in individual chromosomes is taken as the product of the frequencies of the single events. It is evident that multiple aberrations are much less frequent than expected. The low incidence of both the multiple aberrations of similar types, and the multiple aberrations involving both chromosome and chromatid alterations, indicate that random union of broken ends does not occur.

An analysis of multiple aberrations involving alterations induced during the resting stage also indicates that fusions of broken ends of chromosomes are not at random. The data are shown in table 2. In the 1000 cells examined there were 244 centric ring chromosomes and 684 dicentric chromosomes, a ratio of 1 : 2.8 instead of the 1 : 10 ratio expected if fusions of broken chromosome ends were at random. Only the dicentrics have been included in the subsequent analysis of multiple aberrations. Of the 6000 chromosomes analyzed 1270 or 21.2 per cent had only one break, and 49 or 0.8 per cent had two breaks which led to the formation of multicentrics. Random production and fusion of effective breaks should have produced two effective breaks in each of 276 chromosomes or 4.6 per cent. The deficiency of multiple chromosome breaks in the same chromosome is of the same order as the deficiency of multiple aberrations involving either

chromatid or chromosome breaks (table 1). The frequency and types of multicentric chromosomes per cell are more nearly in accord with theoretical expectations. We may assume that fusions can occur between any two of the twelve chromosome arms. With two dicentric bridges per cell we would expect the following combinations: 24 cells with 2 dicentrics, 16 cells with a tricentric chromosome and 1 with a closed dicentric. With 3 dicentric bridges there should be cells with 3 dicentrics, 1 dicentric plus a tricentric, 1 tetracentric, or a closed tricentric, in the ratio of 2 : 12 : 6 : 1. The observed and expected frequencies are shown in table 2. In cells with 2 dicentric bridges there is an excess of dicentrics at the expense of tricentric chromosomes. The deficiency of tricentrics might be expected since one of the three chromosomes involved in the production of a tricentric was effectively broken in each of its two arms, and the previous data show that multiple breaks in the same chromosome occur much less frequently than expected. In cells with three dicentrics the types of aberrations occur with the expected frequency. Apparently with a greater number of effective breaks a more nearly random fusion of broken ends is possible.

The total number of multicentric aberrations per cell also approaches the theoretical frequency. Here the maximum number of aberrations is limited by the number of chromosome arms which may be broken and rejoined in new associations. The calculation of expected frequencies has been done by Dr. Reed. The number of dicentric bridges expected per cell is the total number of cells times the appropriate term of the expansion of $[(1 - q) + q]^n$, where q is the frequency of chromosome arms involved in dicentric aberrations and n is the pairs of chromosome arms. The observed and theoretical frequencies of dicentric bridges are shown in table 2. Since the x^2 test gives a value of $P = 0.02$ it is probable that the deficiency of two or more dicentric aberrations per cell is significant, but the deviation is small compared with deficiencies of expected chromosomes with multiple breaks (table 1).

TABLE 2

FREQUENCY AND TYPES OF MULTICENTRIC CHROMOSOMES PER CELL. DOSE 500 R AT 70 R/M

DICENTRIC BRIDGES	TYPES	NUMBER OBSERVED	FREQUENCY EXPECTED	TOTALS OBSERVED	TOTALS EXPECTED
0	Cell normal	457		457	483
1	1 dicentric	418		418	373
2	2 dicentric	81	66	110	120
	1 tricentric	29	44		
3	3 dicentric	1	1	14	21
	1 di- + 1 tricentric	9	8		
	1 tetracentric	4	4		
4	1 pentacentric	1		1	3

The great deficiency of multiple breaks in a single chromosome of a multicentric complex can be attributed to the inhibiting effects of the first break or to the spatial relations of the chromosomes. If an effective break occurs in a chromosome arm, certain stresses imposed by polarity may be released which inhibit further effective breaks in the same arm. Such an inhibiting mechanism may account, in part, for the low frequency of chromatid aberrations in acentric chromosome fragments. This mechanism would not be expected to operate across the centromere and affect the incidence of effective breaks in the other arm of the same chromosome. This assumption is supported by the fact that ring chromosomes occur much more frequently than expected. The high frequency of ring chromosomes must be attributed to the relatively close association of the two arms of the same chromosome. The low incidence of multiple breaks in a single chromosome involved in multicentric associations can be attributed in part to the spatial relations which prevent simultaneous fusions involving both arms of the same chromosome.

Summary.—The relative frequencies of various types of chromosomal aberrations indicate that secondary factors play an important part in determining the frequency of effective breaks induced by x-rays. These secondary factors include: (1) the spatial relations of the chromosomes, (2) the stresses imposed by the centromere in maintaining polarity and (3) the stage of chromosome development in the nuclear cycle.

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GENETIC CONTROL OF BIOCHEMICAL REACTIONS IN NEUROSPORA: AN "AMINOBENZOICLESS" MUTANT*

By E. L. TATUM AND G. W. BEADLE

DEPARTMENT OF BIOLOGY, STANFORD UNIVERSITY

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Para-aminobenzoic acid has recently been recognized as a factor required for the growth of a number of microörganisms[1] and as a member of the vitamin B group.[2] One of the number of x-ray induced mutants of *Neurospora crassa*, obtained as described elsewhere,[3] is characterized by the loss of ability to synthesize *p*-aminobenzoic acid. This "aminobenzoicless" mutant is differentiated from normal by a single gene, is unable to grow on unsupplemented synthetic medium, but its growth is indistinguishable from normal when *p*-aminobenzoic acid is supplied.

The mutant culture was maintained on "complete" medium containing yeast extract, malt extract and sucrose. Conidia from this stock culture were used for inoculating test cultures. The test medium was the "synthetic" or "minimal" medium previously described[3] containing inorganic salts and nitrogen, sucrose and 4 gammas of biotin[4] per 1000 ml. Agar used in this synthetic medium was freed of *p*-aminobenzoic acid by repeated washing with distilled water. Cultures were incubated at 25°C.

Crosses were made of the *p*-aminobenzoicless mutant (sex *A*, the equivalent of + in Lindegren's terminology[5]) with the normal strain (sex *a*); ascospores were dissected in order from asci and grown on complete medium. These cultures were tested by transferring them to minimal medium and observing for growth. In all, 142 single spore cultures were grown and tested. Of these, 72 proved to be able to synthesize *p*-aminobenzoic acid and 70 were unable to accomplish this. Of 11 asci from each of which all eight ascospore cultures were obtained, all had four normal and four mutant-type spores, and in no case were more than four spores of a kind obtained from any one ascus. The results in the 17 asci in which segregations for both sex and aminobenzoicless could be deduced are summarized in table 1. It is evident from these results that the aminobenzoicless character is inherited as though it were differentiated from normal by a single gene. There is no evidence of linkage of aminobenzoicless and sex. The fact that in 10 out of 17 asci aminobenzoicless segregated from its normal allele in the second division indicates that the aminobenzoicless locus is an appreciable distance from the centromere.

In addition to the original aminobenzoicless mutant (ascospore isolate 1633), which was used for all physiological investigations described except those otherwise noted, an apparently independent occurrence of this mutant was recorded (isolate 5359). A cross between 5359 and 1633-63-6

TABLE 1

SEGREGATION FOR SEX AND ABILITY TO SYNTHESIZE *p*-AMINOBENZOIC ACID, IN ASCI FROM A CROSS OF NORMAL (SEX *a*) AND AMINOBENZOICLESS (SEX *A*)

The base and apex of the ascus are regarded as equivalent and the orientation of second division spindles is disregarded unless this has significance with regard to recombination. Since the third division is equational, each pair of products of this division is reported as a unit.

TYPE OF SPORE ARRANGEMENT				NUMBER OF ASCI OBSERVED
A +	A +	a *pab*	a *pab*	3
A *pab*	A *pab*	a +	a +	4
A +	A *pab*	a +	a *pab*	9*
A +	a +	A *pab*	a *pab*	0
A +	a *pab*	A +	a *pab*	0
A *pab*	a +	A *pab*	a +	0
A +	a *pab*	A *pab*	a +	1

* In two asci the results were such that it was presumed that two spores had been transposed in dissection.

TABLE 2

RESPONSE OF *p*-AMINOBENZOICLESS MUTANT 1633 TO COMPOUNDS RELATED TO *p*-AMINOBENZOIC ACID

Inactive Compounds

Benzoic acid	Sulfanilic acid
o-Aminobenzoic acid	Tyrosine
m-Aminobenzoic acid	2, 4-Diaminotoluene
p-Hydroxybenzoic acid	Acetanilide
p-Chlorobenzoic acid	*p*-Methylcyclohexanol

Active Compounds

SUBSTANCE	ACTIVITY COMPARED WITH THAT OF *p*-AMINOBENZOIC ACID* BY WEIGHT IN LIQUID MEDIUM	BY GROWTH RATE ON SOLID MEDIUM
Acetyl-*p*-aminobenzoic acid	1/50†	
p-Nitrobenzoic acid	1/100	1/120
p-Dimethylaminobenzaldehyde	1/1150‡	1/170‡
p-Toluidine	1/1400	1/1600
Aniline	1/13,000	
p-Nitrotoluene§	1/14,000	1/5000
p-Aminoacetophenone§	1/100,000	

* Activity values based on several series of varied concentrations, and when possible calculated from the concentrations giving $^1/_2$ maximum growth.

† Value obtained by visual comparison with control *p*-aminobenzoic dilutions.

‡ The difference between these two values seems to be correlated with the use of liquid and solid media.

§ Only very slight growth possible with any concentration, figures for activity therefore not too significant.

(derived mutant of sex *a*) gave 68 offspring from single ascospores taken at random, and all of these failed to grow in minimal medium. Presumably

these two aminobenzoicless strains represent independent occurrences of the same gene change. It is improbable that the two strains are the result of mutations of closely linked genes or that the second represents merely a tube contaminated with a spore of the first, although these two possibilities cannot be rigorously excluded.

Physiologically the two aminobenzoic strains are similar if not identical. Although exhaustive measurements of the *p*-aminobenzoic acid require-

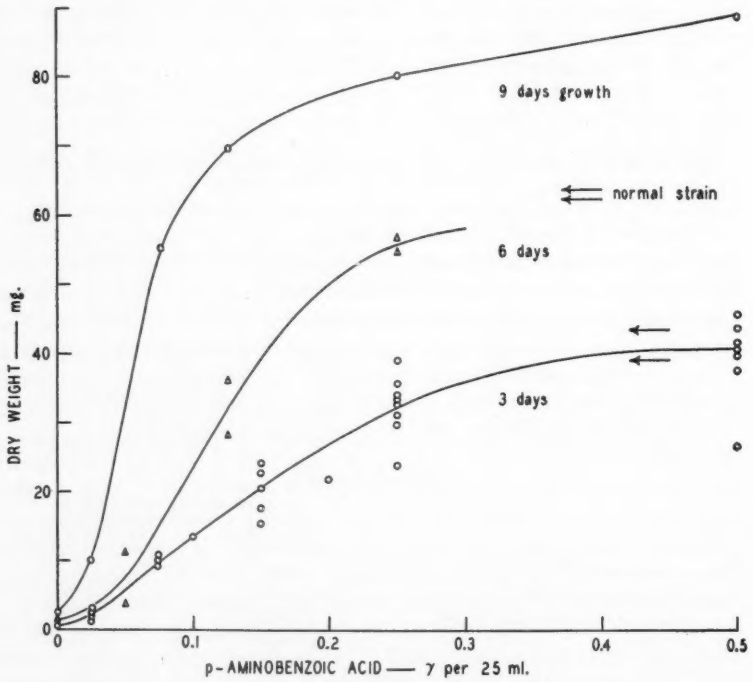

FIGURE 1

Dry weight attained after three, six and nine days as a function of *p*-aminobenzoic acid concentration. Weights of normal strain indicated by arrows.

ment of strain 5359 have not been made, it can be said that this is of the same order of magnitude as that of strain 1633. The quantitative growth responses to the compounds related to *p*-aminobenzoic acid which are listed in table 2 are similar in the two strains. Furthermore they are similarly inhibited by sulfanilamide as indicated in figure 4.

Growth of the aminobenzoicless mutant is normal only in the presence of *p*-aminobenzoic acid. In tests of other known growth factors a slight

but significant response to pimelic acid has been noted. This response is apparent only after a period of five or six days, whereas that to *p*-aminobenzoic acid is evident after one day. The pimelic acid effect has been observed with both liquid and solid media and with carefully recrystallized pimelic acid. This phenomenon, which obviously needs further investigation, suggests either some sort of adaptation or a possible conversion of pimelic acid to *p*-aminobenzoic acid.[6] Whatever its nature may be,

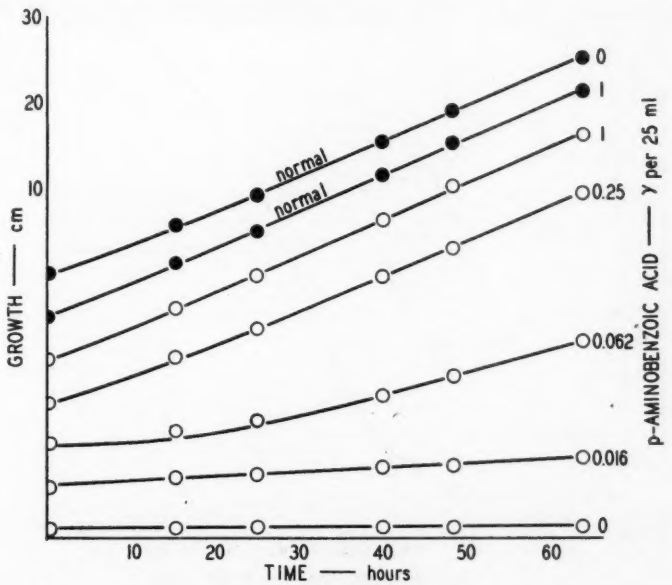

FIGURE 2

Position of mycelial frontiers in growth tubes as a function of time. Results with normal strains indicated by solid circles and those with *p*-aminobenzoicless strain (1633) indicated with open circles. Vertical scale offset for each successive curve in the series.

recovering and retesting strains grown in the presence of pimelic acid shows that the effect does not involve a genetic change in the organism.

The growth of the aminobenzoicless mutant is a function of the amount of *p*-aminobenzoic acid supplied to it. This quantitative relation can be determined either by the increase in dry weight of mycelium in liquid medium, or by rate of progression of the mycelium front through a tube partly filled with agar medium.

For the dry weight determinations, cultures were grown in 250-ml.

Erlenmeyer flasks containing 25 ml. of synthetic medium supplemented with *p*-aminobenzoic acid. Each flask was inoculated with a drop of a water suspension of conidia. At the end of the incubation period, generally 3 days, the mycelium was removed, washed and dried at 105°C. on a weighed watch glass. Figure 1 gives the results of a number of series. The relation between weight and *p*-aminobenzoic acid is almost linear up to a concentration of 0.25 gamma per 25 ml. The average weight reached

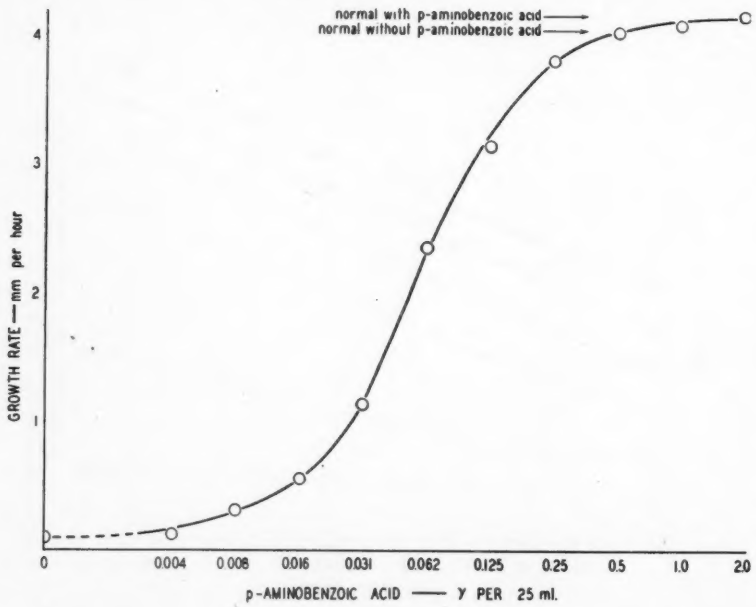

FIGURE 3

Rate of growth in tubes of aminobenzoicless strain (1633) as a function of *p*-aminobenzoic acid content of medium. Rates of normal strains indicated by arrows. Individual points obtained by averaging four independently obtained values.

in 3 days with 0.5 gamma per 25 ml. is about 40 mg., the weight reached by the normal, unsupplemented culture in the same time. The variability is rather great, due to the fact that only one determination can be made for each culture and due to variations in inoculum, length of initial lag period and probably other factors. Because of these difficulties and because significant weights cannot be reached in much less than 3 days, this method is only fairly satisfactory for assay purposes.

A more satisfactory method of determining the relation between *p*-aminobenzoic acid concentration and growth is to measure the rate of

progression of the mycelium front along the surface of agar medium in a horizontal tube as previously described.[3] This method has the advantage that variations inherent in the liquid culture method are not included in the measurements, since the final rate of progression is determined after equilibrium is reached and several measurements may be made in a given culture tube. This method has the further advantage that significant rate measurements may be made over a relatively short period of time, after the rate has become constant. The results of a series of *p*-aminobenzoic acid concentrations are shown in figure 2. As more *p*-aminoben-

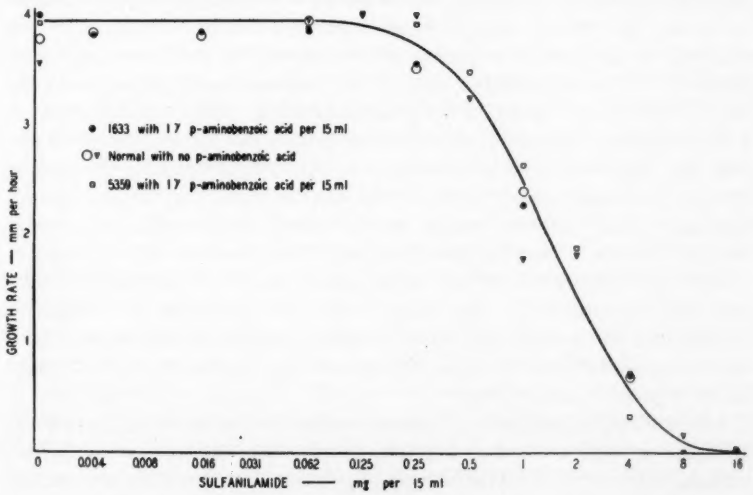

FIGURE 4

Inhibition of normal and aminobenzoicless strains (1633 and 5359) with sulfanilamide. Mutant strains supplied with one gamma *p*-aminobenzoic acid per 15 ml. medium.

zoic acid is supplied the rate of progression increases until with 0.5 gamma per 25 ml. it is the same as that of the normal strain (approximately 4 mm. per hour at 25°C. Figure 3 gives the final relation between the rate and vitamin concentration.

The results of both methods show that when enough *p*-aminobenzoic acid is supplied to the mutant it is indistinguishable from the normal strain. The inability to synthesize this vitamin seems therefore to be the only differentiating factor.

The biological significance of *p*-aminobenzoic acid was first recognized as a result of its action in overcoming the bacteriostatic or inhibitory

effects of sulfanilamide.[7] This antagonism has been interpreted as a competitive reaction, with an excess of sulfanilamide displacing the normally present *p*-aminobenzoic acid from its functional rôle, and an excess of *p*-aminobenzoic acid overcoming the resulting inhibition. The widespread existence of this *p*-aminobenzoic acid—sulfanilamide antagonism has been taken as indicating the essential nature of *p*-aminobenzoic acid for many diverse organisms including diatoms[8] and fungi (trichophyton).[9] Sulfanilamide also completely inhibits the growth of normal and mutant strains of *Neurospora*, and in both cases this inhibition is completely overcome by an excess of *p*-aminobenzoic acid. Figure 4 shows that growth on solid medium is completely inhibited by a sulfanilamide concentration of 16 mg. per 15 ml. In these tests enough *p*-aminobenzoic acid was supplied to the mutant to permit normal growth in the absence of sulfanilamide. The quantitative effect of sulfanilamide was the same on the normal and the mutant strains, demonstrating that the utilization of *p*-aminobenzoic acid rather than its synthesis is blocked by sulfanilamide, and that the amount of *p*-aminobenzoic acid synthesized by the normal strain is not appreciably greater than that required by the mutant for normal growth. Similar results were obtained by measurements of dry weight increase. The effectiveness of added *p*-aminobenzoic acid in overcoming the inhibition by 8 mg. sulfanilamide per 25 ml. in liquid medium was also investigated. The vitamin had the same anti-sulfanilamide activity for the normal and mutant strains, but its activity under these conditions was only about $^1/_{100}$ of its vitamin activity for the mutant strain in the absence of sulfanilamide.

As a working hypothesis, a single gene may be considered to be concerned with the primary control of a single specific chemical reaction.[10] If this premise is accepted, the biosynthesis of *p*-aminobenzoic acid in the mutant strain, which differs from normal by a single gene, should be blocked in only one specific step in the entire complex of reactions. An attempt was made to trace the course of *p*-aminobenzoic acid synthesis and to determine the step that is blocked by the mutant gene. This involved testing a number of substances for their ability to replace *p*-aminobenzoic acid in the growth of the mutant strain.[11] The results obtained by the dry weight and growth rate methods as summarized in table 2 were reasonably consistent with each other. *Ortho-* and *m*-aminobenzoic acids were inactive, as were all the compounds tested which did not have an aromatic N. With the amino group replaced by Cl or OH as in *p*-Cl or *p*-OH-benzoic acids, or as in tyrosine, no activity could be detected. These facts indicate that an aromatic substituted N is essential for activity and that if a second substituent group is present it must be in para position to the N. These results and the inactivity of tyrosine, which is presumably synthesized by the mutant strain, suggest the possibility that the reaction blocked by the

mutant gene may be concerned with the introduction of an amino N into the benzene ring.

If this is the reaction blocked in the aminobenzoicless mutant, normal strains should be able to introduce an amino N into a benzene ring. It is known that normal strains synthesize *p*-aminobenzoic acid or some other active substance, and practically all of the excess of this is contained in the culture medium. It is therefore possible to test such strains for their ability to introduce an aromatic amino group by growing them in the presence of compounds containing a nitrogen-free benzene ring and determining whether the *p*-aminobenzoic acid content of the medium is thereby increased. When grown for 3 days in the absence of such compounds, *N. crassa* produced about 0.1 gamma of *p*-aminobenzoic acid per 25 ml. of medium, indicating that the synthesis of this growth factor is normally not greatly in excess of its needs. This is likewise indicated by the sulfanilamide inhibition data. In the presence of benzoic acid, *p*-hydroxybenzoic acid, or tyrosine, in concentrations up to 1 mg. per 25 ml., the amount of *p*-aminobenzoic acid produced was not detectably increased. In fact, benzoic acid appreciably decreased the amount of *p*-aminobenzoic acid recovered. The conclusion that these compounds are not converted into *p*-aminobenzoic acid is confirmed by the fact that they do not have the anti-sulfanilamide activity to be expected with an increased yield of *p*-aminobenzoic acid.

The failure to detect any conversion of the tested nitrogen-free compounds to *p*-aminobenzoic acid, tends to discredit the suggestion that the mutant gene blocks the introduction of an aromatic amino group. It indicates, rather, that the normal synthesis of *p*-aminobenzoic acid does not involve the introduction of an aromatic N or amino group into a preformed benzene ring. It should be pointed out, however, that the interpretation of all such experiments may be complicated by factors such as penetration into the cells, secondary chemical reactions or the inability to add the possible intermediates in the biological or "active" condition.

A normal precursor of *p*-aminobenzoic acid which is located in the synthetic sequence leading up to the break due to the mutant gene should theoretically be completely inactive for the mutant strain. Similarly, a precursor coming after this break should be comparable in activity to *p*-aminobenzoic acid itself. If all the effective substances listed in table 2 owe their activity to their conversion to *p*-aminobenzoic acid, *Neurospora* must be able to reduce a nitro group,[12] introduce a carboxyl group para to an amino group, oxidize a methyl or aldehyde group and de-acetylate or de-methylate an amino group. The activities of the effective compounds are so much lower than that of *p*-aminobenzoic acid itself, however, that it seems doubtful that any is its normal precursor, or that any of these reactions is involved in the normal synthesis of *p*-aminobenzoic acid.

These compounds may be somewhat active *per se*, or may owe their activity to a conversion to a *p*-aminobenzoic acid by a relatively inefficient mechanism not concerned in the normal biosynthesis of *p*-aminobenzoic acid. This accessory conversion mechanism should be equally efficient in the normal and mutant strains. In either case the compounds should have the same anti-sulfanilamide activity for both strains if they are not normal precursors of *p*-aminobenzoic acid. Of all the active compounds listed (table 2), only *p*-nitrobenzoic acid and aniline had any anti-sulfanilamide activity,[13] and no quantitative difference in their effect on the normal and mutant strains was observed.

It may be concluded that the active compounds listed in table 2 are probably not concerned in the normal synthesis of *p*-aminobenzoic acid. In this biosynthesis the N or amino group is probably introduced before the formation of the benzene ring.

Summary.—An x-ray induced mutant strain of *Neurospora crassa* has been obtained which requires *p*-aminobenzoic acid for growth. Its growth is a function of the amount of *p*-aminobenzoic acid supplied, and it is indistinguishable from normal when adequate amounts of *p*-aminobenzoic acid are available.

The mutant differs from normal by a single gene, which must therefore control an essential step in the synthesis of *p*-aminobenzoic acid, and which is presumably primarily concerned only with the synthesis of *p*-aminobenzoic acid.

Sulfanilamide inhibits the growth of both the normal and mutant strains, and in both cases the inhibition is overcome by an excess of *p*-aminobenzoic acid.

A number of substances related to *p*-aminobenzoic acid are able to replace it, but their activities are much less than that of *p*-aminobenzoic acid itself.

The addition of benzoic or *p*-OH-benzoic acids or tyrosine did not increase the amount of *p*-aminobenzoic acid produced by the normal strain.

It is concluded that none of the compounds tested is concerned with the normal synthesis of *p*-aminobenzoic acid, and that this biosynthesis probably does not involve the introduction of an amino group into a preformed benzene ring.

* Work supported by grants from the Rockefeller Foundation and from the Penrose Fund of the American Philosophical Society. The authors are indebted to Dr. Russell Perry Hager and Miss Caryl L. Parker for assistance.

[1] See Fildes, P., *Lancet*, **238**, 955 (1940); Rubbo, S. D., and Gillespie, J. M., *Nature*, **146**, 838 (1940); and Lampen, J. O., and Peterson, W. H., *Jour. Am. Chem. Soc.*, **63**, 2283 (1941).

[2] Ansbacher, S., *Science*, **93**, 164 (1941); and Sure, B., *Ibid.*, **94**, 167 (1941).

[3] Beadle, G. W., and Tatum, E. L., *Proc. Nat. Acad. Sci.*, **27**, 499 (1941).

[4] S. M. A. Corporation, Chagrin Falls, Ohio. Biotin concentrate 1000.

[5] Dodge originally used "*A*" and "*a*" to designate the two sex types in *Neurospora crassa*. Lindegren later substituted the symbols "+" and "−" for these. Because of the desirability of using the symbol + for the normal allele of a gene and also to designate a positive experimental result, we prefer Dodge's original designations.

[6] Neither conversion of pimelic acid to *p*-aminobenzoic acid by the normal strain nor anti-sulfanilamide activity of pimelic acid could be detected.

[7] See Woods, D., and Fildes, P., *Chem. Ind.*, **59**, 133 (1940); Woods, D., *Brit. Jour. Exp. Path.*, **21**, 74 (1940); and Landy, M., and Wyeno, J., *Proc. Soc. Exp. Biol. Med.*, **46**, 59 (1941).

[8] Wiedling, S., *Science*, **94**, 389 (1941).

[9] Dimond, N. S., *Ibid.*, **94**, 420 (1941).

[10] See Beadle, G. W., and Tatum, E. L., *Amer. Nat.*, **75**, 107 (1941), and loc. cit., footnote 3.

[11] All the compounds tested were carefully purified to remove any possible traces of *p*-aminobenzoic acid. The aniline was prepared by hydrolysis of recrystallized acetanilide and subsequent distillation.

[12] A bacteriostatic action of *p*-nitrobenzoic acid has been reported. See King, J. T., and Henschel, A. F., *Proc. Soc. Exp. Biol. Med.*, **47**, 400 (1941).

[13] The other active compounds were toxic in concentrations theoretically high enough to overcome the sulfanilamide inhibition.

THE DISSOCIATION CONSTANT IN NITROGEN FIXATION BY AZOTOBACTER

BY P. W. WILSON, R. H. BURRIS AND C. J. LIND

DEPARTMENT OF AGRICULTURAL BACTERIOLOGY, UNIVERSITY OF WISCONSIN

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The dissociation constant of nitrogen fixation by *Azotobacter* was first estimated by Lineweaver, Burk and Deming[1] from their data on the influence of the $p\mathrm{N}_2$ on rate of fixation by this organism. These workers measured fixation in the Warburg respirometer by observing the increase in the rate of oxygen uptake with time. The initial steps of nitrogen fixation by this organism were formulated as:

$$E + \mathrm{N}_2 \rightleftarrows \mathrm{N}_2E \text{ (rapid)} \qquad (1)$$

$$\mathrm{N}_2E \rightarrow E + P \text{ (slow)} \qquad (2)$$

where E = Enzyme (nitrogenase) concerned with first step of fixation,

P = Products (increase in *Azotobacter* cells).

Using the method of Lineweaver and Burk,[2] they calculated a Michaelis constant (K_{N_2}) for reaction 1 of 0.21 atm. In their experiments the $p\mathrm{O}_2$ was kept constant at 0.2 atm. and hydrogen added to bring the total pressure to one atmosphere whenever the $p\mathrm{N}_2$ was less than 0.8 atm. Since

Wilson and his collaborators[3, 4] subsequently demonstrated that molecular hydrogen competitively inhibits both symbiotic and asymbiotic nitrogen fixation, this estimate of the K_{N_2} must be discarded.[5, 6]

Wyss, *et al.*,[6] have recently calculated a preliminary value for K_{N_2} based on experiments in which hydrogen was not used. The major purpose of their experiments, however, was to compare the nitrogen fixing system in *Azotobacter* with that in inoculated red clover plants. Consequently, they employed the same technique for preparation and handling of the gas mixtures as had been used previously in tests with the symbiotic system. The pN_2 in the mixtures was probably accurate to no more than 0.01 atm. even at the lower partial pressures of this gas. Although great precision was not claimed for the estimate, Wyss and his associates believed the K_{N_2} value was near 0.01 to 0.02 atm. Recently we have checked this estimate through use of a number of methods, several of which have not been previously described.

Fixation at pN_2 0.2 and 0.78 Atm.—During the symposium on respiratory enzymes held at the University of Wisconsin in September, 1941, the senior author discussed with Dr. Dean Burk the question of the probable value for the Michaelis constant of nitrogen fixation. In the course of our discussions and subsequent correspondence he pointed out that our published data indicated that the rate of fixation (as measured by the monomolecular velocity constant, k) at a pN_2 of 0.2 atm. did not differ significantly from that in air. He suggested that a difference should be detectable unless the Michaelis constant was rather small since the familiar Michaelis-Menten equation states

$$k/k_{max.} = pN_2/(pN_2 + K_{N_2}). \qquad (3)$$

Substitution of possible values for K_{N_2} into this equation leads to the following:

K_{N_2}	$k/k_{max.}$ 0.2 ATM.	$k/k_{max.}$ AIR	$\frac{k \text{ AT } 0.2 \text{ ATM.}}{k \text{ IN AIR}}$
0.01	95.3%	98.7%	96.5%
0.02	90.9%	97.5%	93.2%
0.03	87.0%	96.3%	90.3%

This observation suggested to us a method for determining the probable limits of K_{N_2} provided that reliable estimates of the error in the k values were available. Our values of the k's had been calculated from the slopes of straight lines by the usual statistical procedures; hence their errors were known. For the macronitrogen experiments each value of k was based on six observations. From 25 such lines the average error in the estimate of k was calculated as 5.72 ± 0.41 per cent. Even though the K_{N_2} were as much as 0.03 atm., a *significant* difference would not always be obtained

in an *individual* experiment. If, however, a large number of experiments were combined, a real difference should be evident, and its magnitude should indicate the probable size of K_{N_2}. Data from 18 experiments were available. The mean value for k in air was 0.0715, in a pN_2 of 0.2 atm., 0.0688; the mean difference was 0.0027 ± 0.0009. The k values for the pN_2 of 0.2 atm. ranged from 87 to 108 per cent of the corresponding value in air with an average of 96.7 ± 1.23. This value suggests a Michaelis constant of 0.01 but is consistent with one as high as 0.02 atm.

The microrespirometer method offers the advantage that a large number of determinations can be readily made for estimation of k with consequent reduction in its error. Whereas it is feasible to base the k values on only 6 determinations in the macronitrogen experiments, duplicate flasks on which 10 readings are taken over a period of 5 hours are easily handled by the microrespirometer technique. In the first instance the k value has 4 degrees of freedom, in the second, 16; a substantial reduction in the error of estimate would be expected. The average error in 22 such estimates of k from microrespiration experiments was 3.71 ± 0.24 per cent. Our previous studies included just three trials with a pN_2 of 0.2 atm., and the k values from these had only 9–12 d. f. None was significantly different from the air control; the k values from a large number of trials with a pN_2 of 0.3 atm. also were identical within experimental error with those in air. Three new experiments were made in which triplicate flasks of each treatment were taken and 10 readings made on each. The mean values of the velocity

TABLE 1

EFFECT OF pN_2 ON REACTION VELOCITY CONSTANT IN MICRORESPIRATION EXPERIMENTS

$p'N_2$ IN ATM.	k	k'	$k - k'$	K_{N_2}
0.214	0.245 ± 0.0080	0.219 ± 0.0072	0.026 ± 0.011	0.037 ± 0.017
0.019		0.120 ± 0.0055	0.125 ± 0.009	0.021 ± 0.0023
0.214	0.233 ± 0.0099	0.219 ± 0.0076	0.014 ± 0.012	0.020 ± 0.018
0.019		0.095 ± 0.0054	0.138 ± 0.011	0.029 ± 0.0037
0.214	0.242 ± 0.0049	0.215 ± 0.0082	0.027 ± 0.009	0.039 ± 0.014
0.019		0.090 ± 0.0099	0.152 ± 0.011	0.034 ± 0.0065

k, velocity constant in air; k' in indicated $p'N_2$.

constants were thus based on 24 d. f. so that a difference of about 8% in two means would be significant. The results in table 1 show that in two experiments a significant difference was evident; in the other, the difference was in the right direction but did not exceed experimental error.

Calculation from Formula.—If k represents the rate constant for fixation in air and k' that at some other partial pressure of nitrogen, $p'N_2$, the following relation can be derived from equation (3):

$$K_{N_2} = \frac{k - k'}{k'/p' - k/p}. \qquad (4)$$

TABLE 2

THE $p\mathrm{N}_2$ FUNCTION OF NITROGEN FIXATION BY *Azotobacter* IN MICRORESPIRATION EXPERIMENTS†

$p\mathrm{N}_2$ IN ATM.	EXPERIMENT 1		EXPERIMENT 2		EXPERIMENT 3	
0.01	0.139 0.113	0.126	0.107 0.098	0.103	0.146 0.139	0.142
0.015	0.131 0.130	0.130			0.155 0.156	0.155
0.025	0.173 0.171	0.172	0.173 0.167	0.170	0.202 0.209	0.205
0.05	0.192 0.181	0.186	0.184 0.166	0.175	0.230 0.223	0.227
0.10	0.232 0.212	0.222	0.197 0.185	0.191	0.246 0.242	0.244
0.20	0.234 0.230	0.232	0.216 0.202	0.209	0.253 0.259	0.256
0.78			0.204 0.235	0.220		
Significant difference*	0.017		0.015		0.013	

* Difference between means necessary for significance.

† Values in the table are reaction velocity constants—the k values.

Values of K_{N_2} given in table 1 were calculated from this formula. In addition to the experiments already described, parallel flasks were included in which the $p\mathrm{N}_2$ was 0.019 atm. This atmosphere was prepared by combining 0.8 atm. of tank helium with 0.2 atm. of pure oxygen. According to the manufacturer, the tank helium contains about 1.8% N_2; this value had been used in our previous experiments. For these experiments, however, more precise control of the $p\mathrm{N}_2$ was believed essential so the nitrogen content in the particular tank of helium used was determined by an application of the isotope dilution principle.[7] Known quantities of tank helium and nitrogen gas containing an excess of the N^{15} isotope were mixed, and the isotope dilution effected by the normal nitrogen in the tank helium was measured by analysis in the mass spectrometer. Using two widely different mixtures, we obtained values of 2.29 and 2.46% N_2 in the helium. The values of the K_{N_2} calculated from these data were definitely higher than those usually obtained. It should be observed, however, that, except for one case, the high values have a very large error* so that they are not inconsistent with a K_{N_2} of about 0.02 atm.

As has been mentioned, no special precautions were taken in our previous experiments to control [within narrow limits] the $p\mathrm{N}_2$ in the atmospheres. The effect of this source of error would be increased as the $p\mathrm{N}_2$ was decreased. When the latter exceeded 0.05 atm., a variation 0.01 would not markedly alter the estimate of K_{N_2}. Accordingly, values of this con-

stant were calculated by equation (4) from all trials in which a pN_2 of 0.05 to 0.2 atm. had been used. The results were:

TYPE OF EXPERIMENT	NUMBER	K_{N_2}
Microrespirometer	16*	0.029 ± 0.0020 atm.
Macronitrogen (Wyss)	12	0.013 ± 0.0014
Macronitrogen (Lind)	16	0.020 ± 0.0025

* Includes the 6 trials in table 1.

The pN_2 Function.—As check on these values we carried out new experiments in which elaborate precautions were used to control the pN_2 in the

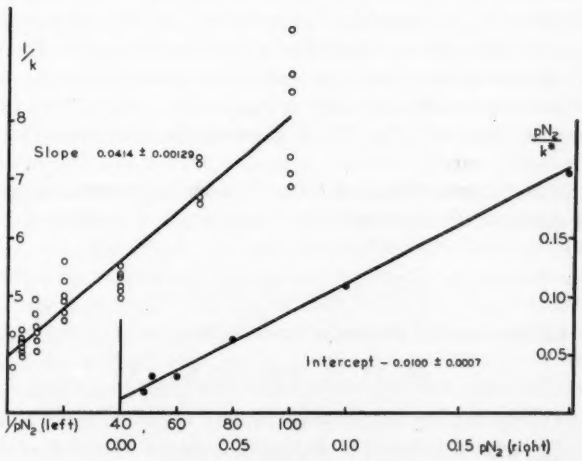

FIGURE 1

Estimation of K_{N_2} by methods based on equations (5) and (7).

(Data of microrespiration experiments. Since the scatter about the line was too small to allow plotting of individual points, only the mean values are shown in line at right; all values are plotted for line at left. The apparent worse fit of these points to the "best" line arises primarily from difference in scale.)

atmospheres. A source of pure helium was used (100% according to manufacturer), and the required atmospheres prepared over mercury with a Toepler pump using pure oxygen generated from potassium chlorate. The gases were transferred to containers which had been evacuated to 10^{-3} mm. Hg. The Warburg flasks were evacuated to 25 mm. Hg residual pressure and pure oxygen added; this was repeated three times, then the desired gas mixture added after the fourth evacuation. Water, autoclaved just before use, served as the displacing fluid. In spite of every care, it is believed that the nitrogen content in the atmospheres supplied the azoto-

bacter was slightly higher than calculated since helium-oxygen controls showed a small but definite increase in respiration with time. There was no increase in the respiration of the hydrogen-oxygen controls in which fixation would not occur even though a small quantity of nitrogen accidentally did get into the mixture. The estimates of K_{N_2} based on the data in table 2, therefore, are probably low and should be regarded as minimum values.

One estimate of the K_{N_2} was made from these data by the method of Lineweaver and Burk[2] based on the equation:

$$1/k = (K_{N_2}/k_{max.})(1/pN_2) + 1/k_{max.}. \quad (5)$$

The value for $1/k_{max.}$ (intercept) was determined for each experiment, then the individual values adjusted to a common intercept of 4.0. The combined data were fitted to a line with this intercept, and the K_{N_2} estimated by dividing the slope by 4.0; test of the line showed no evidence of departure from linearity (Fig. 1). A value of 0.0104 ± 0.00032 atm. was obtained.

A second method for estimating K_{N_2} from such data was suggested by Burk (personal communication). If equation (5) is multiplied by (pN_2) it becomes:

$$pN_2/k = pN_2/k_{max.} + K_{N_2}/k_{max.}. \quad (6)$$

If $k_{max.}$ is taken as unity, this equation reduces to:

$$pN_2/k^* = pN_2 + K_{N_2} \quad (7)$$

in which k^* is the velocity constant relative to $k_{max.} = 1.00$. In each experiment $k_{max.}$ was determined by the method based on equation (5), then *relative k* values calculated from the observed k's by dividing each by the proper $k_{max.}$. Values of pN_2/k^* were plotted against the pN_2 and a straight line with unit slope fitted to the points. The intercept, 0.0100 ± 0.0007, determines K_{N_2} (Fig. 1).

Inhibition Experiments.—Burk[5] observed that the slope/intercept from the lines based on the experiments in which hydrogen was the replacing gas did not determine K_{N_2} as originally supposed but a more complex relationship:

$$K_{H_2}/K_{N_2} = 5(1 + K_{H_2}) \text{ or} \quad (8)$$

$$= 5/(1 - 5K_{N_2}). \quad (9)$$

Since K_{N_2} is likely between 0.01 and 0.02 atm., the value of K_{H_2}/K_{N_2} is close to 5.4; also moderate change in K_{N_2} would not markedly alter the value of the ratio. From the equation of competitive inhibition by hydrogen,[4] the following relationship is derived:

$$K_{N_2} = \frac{pH_2/(K_{H_2}/K_{N_2}) - pN_2(k/k_i - 1)}{(k/k_i - 1)} \qquad (10)$$

in which at a given pN_2, k and k_i are the rates in the absence and presence of hydrogen, respectively. Ten experiments were available which allowed test of this equation. As the error of the estimate was rather large, it was not surprising that the calculated values of K_{N_2} fell on either side of zero. Three experiments were discarded since the estimates of K_{N_2} were obviously too large—order of ± 0.10 atm. The mean of the remaining seven was 0.014 ± 0.0066 atm.

Discussion.—In evaluating the estimates of K_{N_2} from the several types of experiments two sources of error must be distinguished—experimental errors in the determinations and absolute errors caused by the particular technique used. The first of these has been taken care of by the statistical treatment of the data. Six to thirty determinations were made in each trial, and several experiments were combined for the final estimate. As a result the experimental error was quite small except that based on the inhibitor experiments. Sources of possible absolute error are more difficult to recognize and eliminate. One has been discussed in the text: the variation in the pN_2. A second is that the velocity constant of fixation is indirectly measured in the microrespiration experiments. Although nitrogen fixed and increase in oxygen uptake undoubtedly roughly parallel each other, assurance is lacking that the relationship remains linear as the experimental conditions change, e.g., as the pN_2 is altered. In the macronitrogen experiments secondary effects arising from aging of the culture may influence the results. The fact that a constant rate is obtained when the logarithm of the nitrogen fixed is plotted against time suggests, however, that in the 24 to 36 hours required for these trials, such effects were not marked.

Consideration of all estimates of K_{N_2} suggests 0.02 ± 0.005 atm. as the most probable value. The estimate from the most precise experiments was 0.01 atm., but there is reason to believe that this is a minimum figure. The highest estimate was 0.029 atm. Since this value is based on experiments in some of which no special care was taken to control the pN_2 (variation of at least 0.01 atm.), it probably represents a maximum. The remaining three estimates are consistent with 0.02 atm. We are inclined to the view that the lower limit of the suggested value, i.e., 0.01 to 0.015 atm., is more probable than is the indicated upper limit 0.025 to 0.03 atm., since the very high values were observed only in microrespiration experiments in which the measurement of fixation is indirect. Some variation would be expected because of the different methods used. The relatively close agreement among the figures from different types of experiments and methods of estimation lends confidence to the results and to the mechanism

hypothesis on which they are based. If 0.02 atm. is taken as most likely for the K_{N_2}, then K_{H_2} becomes 0.11 ± 0.028 atm., a value in good agreement with that previously made by Wyss, *et al.*[6]

Summary.—Four methods for estimating the dissociation (Michaelis) constant of an enzyme-substrate complex are illustrated with data from experiments on nitrogen fixation by *Azotobacter vinelandii*. The values found for this reaction ranged from 0.01 to 0.029 atm.; based on present results, 0.02 ± 0.005 atm. is suggested as the most probable estimate. The corresponding value for the K_{H_2}, i.e., for the dissociation constant of the enzyme-inhibitor complex, is 0.11 ± 0.028 atm.

The authors express their appreciation to Dr. Dean Burk for his helpful suggestions and especially for permission to use the method based on equation (7). We are also indebted to Dr. Churchill Eisenhart, Station Statistician, for advice on the several statistical procedures used including the method for estimating the correlation between $(k - k')$ and $(k'/p' - k/p)$, i.e., a and b. The mass spectrometric analyses were made by F. J. Eppling through the courtesy of Professor H. B. Wahlin of the Physics department.

* Note that K_{N_2} is the ratio of two quantities, a/b, each subject to errors which are correlated as follows: $r = -(p'\sigma_k^2 + p\sigma_{k'}^2)/(pp'\sigma_a\sigma_b)$. The estimate of $K_{N_2} = a/b(1 - r\sigma_a\sigma_b/ab + \sigma_b^2/b^2)$, as well as its error, was determined by the method described by Yule[8] for determination of mean and standard deviation of an index.

[1] Lineweaver, H., Burk, D., and Deming, W. E., *Jour. Amer. Chem. Soc.*, **56**, 225–230 (1934).

[2] Lineweaver, H., and Burk, D., *Ibid.*, **56**, 658–666 (1934).

[3] Wyss, O., and Wilson, P. W., *Proc. Nat. Acad. Sci. (U. S.)*, **27**, 162–168 (1941).

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SOME DEDUCTIONS FROM FROBENIUS'S THEOREM

BY G. A. MILLER

DEPARTMENT OF MATHEMATICS, UNIVERSITY OF ILLINOIS

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In 1895 G. Frobenius announced the theorem that if m is a factor of the order of a finite group G then the number of the operators of G, including the identity, whose orders divide m is a multiple of m. A necessary and sufficient condition that G is cyclic is that this number is equal to m for every divisor of the order of G. This results directly from the facts that every subgroup of a cyclic group is cyclic and that a cyclic group contains one and only one subgroup whose order is an arbitrary divisor of the order of the group. Whenever G is non-cyclic its order is divisible by at least one positive integer such that the number of the operators of G whose orders divide this integer exceeds it. This number is clearly not equal to unity or to the order of G. In the particular case when G is a dihedral group it is easy to see that whenever a factor of the order of G is odd then G contains exactly as many operators whose orders divide this factor as there are units in the factor but whenever such a factor is even and less than the order of G then the number of the operators of G whose orders divide this factor exceeds it.

To illustrate this theorem of Frobenius it may be desirable to prove it separately for the case when G is any abelian group. This may also throw additional light on the characteristic subgroups of this important class of groups. It is obvious that every abelian group contains at least one subgroup whose order m is an arbitrary divisor of the order of the group. Since the order of a subgroup is divisible by the order of each of its operators it results that when G is abelian and m is a divisor of the order of G then G contains at least m operators whose orders divide m. A necessary and sufficient condition that G contains no other operator whose order divides m is that this subgroup of order m is composed of all the operators of G whose orders divide m. In general, all such operators constitute a special characteristic subgroup of G whose order is a multiple of m because the order of a group is a multiple of the order of each of its subgroups. This proves the theorem of Frobenius for abelian groups.

It is not difficult to determine the number of the characteristic subgroups of the abelian group G which constitute the given category. In the special case when the order of G is a power of a prime, say p^α, the number of these subgroups is clearly $\beta + 1$ if p^β is an operator of highest order contained in G and the identity and the group itself are included among the characteristic subgroups. In the general case the order of G may be assumed to be $p_1^{\alpha_1} p_2^{\alpha_2} \ldots p_k^{\alpha_k}$, $p_1, p_2, \ldots, p_k$ being distinct prime numbers.

If we let $p_1^{\beta_1}$, $p_2^{\beta_2}$, . . ., $p_k^{\beta_k}$ be the orders of the largest operators in the Sylow subgroups of G then the number of the given characteristic subgroups of G, including the group itself and the identity, is $(\beta_1 + 1)$ $(\beta_2 + 1)$ . . . $(\beta_k + 1)$. Hence *this is the number of the factors of the order of G which have the property that they are equal to the number of the operators of G whose orders divide them respectively, whenever G is abelian.* For all other factors of the order of G there are more operators of G whose orders divide these factors than there are units in these respective factors.

Suppose now that G contains a cyclic Sylow subgroup of order p^m. If such a G contains a subgroup of index p this subgroup is composed of all the operators of G whose orders are not divisible by p^m since it is then generated by these operators and any one of its operators of order p^m. In every case the number of the operators of G whose orders are not divisible by p^m cannot exceed the order of G multiplied by $(p - 1)/p$ according to the theorem noted at the opening of this article since this number must be a multiple of the order of G divided by p and cannot be equal to the order of G. In the particular case when $p = 2$ it is obviously equal to this number and it is then easy to see that these operators constitute a subgroup of index 2 under G since they are composed of the positive permutations of G when G is represented as a regular permutation group. These positive permutations then constitute an invariant subgroup of index 2 under G. In the special case when the order of G is twice an odd number this reduces to the well-known theorem that the operators of odd order in such a group constitute an invariant subgroup under the group.

Every transitive permutation group of degree n is known to have an order which is a multiple of n since it contains a subgroup of index n composed of all its permutations which omit a given letter. When the group is non-regular its order is therefore always divisible by a number which exceeds unity and is less then the order of the group. The number of its permutations whose orders divide this number must therefore be a multiple of the order of this subgroup composed of all its permutations which omit a given letter according to the theorem under consideration. In the special cases when G is either symmetric or alternating this theorem can readily be proved directly. In the former case when n is composite and exceeds 4 it is easily seen that all the permutations of the group have orders which divide $(n - 1)!$ since every substitution of degree n contained therein has this property. When n is a prime number p then the number of the permutations of degree p is obviously $(p - 1)!$ and their orders do not divide $(n - 1)!$

Hence it results that in the symmetric group of degree m all the operators have orders which divide the order of its subgroup composed of all its permutations which omit a given letter whenever n is composite and exceeds 4. When n is prime then all of these operators except $(n - 1)!$ have this

property. This is also clearly the case when $n = 4$. When G is the alternating group of degree n and n is a composite number greater than 4 it follows for the same reason as in the case of the symmetric group that the orders of all its operators divide the order of its subgroup of index n, but when n is a prime number there are twice as many operators in the alternating group whose orders do not divide the order of its subgroup of index n as there are operators in this subgroup since the number of these operators is the same as in the case of the corresponding symmetric group but the order of the subgroup is only half as large.

If in an odd prime power group the number of the operators whose orders divide one of the factors of the order of the group which exceeds unity but is less than the order of the group is exactly equal to this factor then this is also true as regards the number of operators whose orders divide any other such factor. This results directly from the known fact that such a group is necessarily cyclic when it satisfies the given condition because it contains one and only one proper subgroup of every order which exceeds unity and is less than the order of the group.[1] Similar remarks apply to the groups of order 2^m except that in this case the given factor must exceed 2 since the dicyclic group of order 2^m contains only one subgroup of order 2. It is obvious that a necessary and sufficient condition that a group of order p^m, p being a prime number, is cyclic is that it contains operators whose orders do not divide p^{m-1}.

A necessary and sufficient condition that the group G contains an invariant Sylow subgroup of order p^m is that it contains exactly p^m operators whose orders are powers of p. If it contains more than p^m such operators it contains at least p^{m+1} such operators since it must then contain at least $p + 1$ Sylow subgroups of order p^m and if it contains more than $p + 1$ such subgroups it also contains more than p^{m+1} operators whose orders are powers of p. According to the given theorem of Frobenius it must then contain $p^{m+1} + kp^m$ operators whose orders are powers of p. The number of these operators diminished by unity is obviously divisible by $p - 1$ and hence k is divisible by $p - 1$. That is, the number of the operators of G whose order is a power of p is $(1 + l)p^{m+1} - lp^m$, where $k = l(p - 1)$. When $l = 1$ this reduces to $2p^{m+1} - p^m$, which is therefore the smallest number of operators in G whose orders are a power of p whenever this number exceeds p^{m+1}.

From the theorem noted at the opening of this article it results directly that the number of the operators of a group whose orders do not divide a factor of the order of a given group is either zero or a multiple of this factor. This form of the theorem is sometimes useful when it is employed in determining the total number of the abstract group of a given order. In particular, when G is a group of order 12 then the number of its operators whose orders divide 4 is either 4 or 8 since not all of its operators can have

orders which divide 4 in view of Sylow's theorem. For the same reason the number of its operators whose orders do not divide 4 is either 8 or 4. Hence a group of order 12 has either 4 or 8 operators whose orders are divisible by 3. If it contains 8 such operators it must involve four subgroups of order 3 and hence it must be the alternating group of order 12 since it transforms these subgroups transitively. If it has only 4 such operators it must involve the cyclic group of order 6 as an invariant subgroup and hence if it is abelian it is either cyclic or the direct product of the cyclic group of order 6 and the group of order 2. If it is non-abelian it is either dihedral or dicyclic. The determination of the five possible groups of order 12 results therefore very easily from the given theorem. This is also true of the fifteen possible groups of order 24.

[1] Miller, Blichfeldt, Dickson, *Finite Groups*, p. 128 (1916).

ON MONOTHETIC GROUPS

By Paul R. Halmos and H. Samelson

The Institute for Advanced Study

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§ 1. A topological group G is called *monothetic* (following van Dantzig[1]) if there exists a cyclic subgroup H which is dense in G, i.e., the closure of H is G. A generating element of such a cyclic subgroup is called a *generator* of G.

All groups considered in the sequel are abelian; monothetic groups are evidently abelian.

The elements of finite order of a discrete group K form a subgroup, the *torsion group* $T(K)$ of K.

We call a group G *separable* if there exists a countable subset which is dense in G.

We use the theory of character groups. We denote by C the value group for the characters, the group of real numbers mod 1 with the usual topology; let $\overline{C}$ be the same group, but with the discrete topology. We denote by h the natural mapping of $\overline{C}$ on C; it is continuous and an algebraic (but not of course topological) isomorphism.

The character group of a group G is denoted by G^*. The *annihilator* of a subset H of G is the set of those characters of G which map every element of H into the zero element of C; it is a (closed) subgroup of G^*; we denote it by $A(H)$. We recall that the character group of a compact (discrete) group is discrete (compact); for both types of groups we have

the duality theorem, which says $G^{**} = G$, and more generally, $A(A(H)) = H$ for every closed subgroup H.

§ 2. We restrict our considerations to locally compact groups (as is customary in the theory of abelian groups). It is known that a locally compact monothetic group is either compact or discrete.[2] The discrete case being trivial we consider only the compact case. We prove

THEOREM I. *A compact group G is monothetic if and only if its character group G^* is isomorphic to a subgroup of $\overline{C}$.*

Proof. (*a*) Suppose G is monothetic; let d be a generator of G. Let $f = f(x)$ $(x \in G, f(x) \in C)$ be an arbitrary character of G; the mapping $f \to h^{-1}(f(d))$ is obviously an isomorphism of G^* into $\overline{C}$, because every character f is completely determined by its value $f(d)$ for the generator d of G.

(*b*) Suppose G^* is a subgroup of $\overline{C}$. The mapping h of $\overline{C}$ into C induces a homomorphic mapping of G^* into C, i.e., a character of G^*; call this character d. Since $G = (G^*)^*$, d may be considered as an element of G. Let D be the subgroup of G generated by d, i.e., the closure of the cyclic group generated by d. The character d maps only the zero element of G^* into the zero of C; this means that the annihilator of d, and hence also that of D, contains only the zero of G^*. But this means obviously that D equals G. This proves Theorem I.

(*c*) From the considerations in (*a*) and (*b*) it is clear that an element d of G is a generator of G if and only if its annihilator contains only the zero element of G^*.

(*d*) Suppose again G^* is a subgroup of $\overline{C}$. It can be shown easily by elementary methods, without using the duality theorem, that the cyclic group generated by the character d of G^*, defined in (*b*), is dense in the character group G^{**} of G^*, by direct consideration of the neighborhoods of an arbitrary element of G^{**}.

§ 3. THEOREM II. *A discrete group H is isomorphic to a subgroup of $\overline{C}$ if and only if its power (cardinal number) is $\leqq \mathfrak{c}$ (= the power of the continuum) and its torsion group $T(H)$ is isomorphic to a subgroup of $\overline{C}$ and so of $T(\overline{C})$.*

Theorem II is a consequence of the following

LEMMA. *Let H be a discrete group of power $\leqq \mathfrak{c}$; every isomorphic mapping f_1 of $T(H)$ into $\overline{C}$ can be extended to an isomorphic mapping f of H into $\overline{C}$.*

Proof. The complement of $T(\overline{C})$ contains $\mathfrak{c}$ linearly independent elements (elements of a Hamel basis for the real numbers, reduced mod 1); let $\lambda_1, \lambda_2, \ldots$ be a well ordering of these elements. We well order also the elements of the complement of $T(H)$: $x_1, x_2, \ldots$. We consider the groups H_α generated by the elements of $T(H)$ and the x_β with $\beta < \alpha$, and construct isomorphic mappings f_α of H_α into $\overline{C}$ such that f_α is an extension of f_β for $\beta < \alpha$. We start with the given f_1. Suppose f_β is constructed for $\beta < \alpha$.

(*a*) If α is a limit ordinal then H_α is the union of the H_β with $\beta < \alpha$.

Every $z \in H_\alpha$ is contained in some H_β, $\beta < \alpha$; we define $f_\alpha(z) = f_\beta(z)$. It is clear that this f_α has the desired properties.

(b) If α is not a limit ordinal then H_α is generated by $H_{\alpha-1}$ and $x_{\alpha-1}$.

(b') If no multiple of $x_{\alpha-1}$ belongs to $H_{\alpha-1}$, then H_α is the direct sum of $H_{\alpha-1}$ and the cyclic group generated by $x_{\alpha-1}$; we extend $f_{\alpha-1}$ by putting $f_\alpha(x_{\alpha-1}) = \lambda_{\alpha-1}$. This gives an isomorphic extension, because under our constructions λ_γ and the elements of the groups $f_\beta(H_\beta)$ with $\beta \leqq \gamma$ are always linearly independent.

(b'') If a multiple of $x_{\alpha-1}$ belongs to $H_{\alpha-1}$, let n be the smallest positive integer for which $nx_{\alpha-1} \in H_{\alpha-1}$. Every element z of H_α can then be written in a unique manner as $y + mx_{\alpha-1}$ with $y \in H_{\alpha-1}$ and $0 \leqq m < n$. Let λ be an element of $\overline{C}$ with $n\lambda = f_{\alpha-1}(nx_{\alpha-1})$. Put $f_\alpha(z) = f_{\alpha-1}(y) + m\lambda$. It is easily verified that f_α is a homomorphic mapping of H_α, and that it is an extension of $f_{\alpha-1}$. To prove that it is an isomorphism, suppose $f_\alpha(z) = 0$. We have then $nz = 0$, because $nz \in H_{\alpha-1}$, $f_{\alpha-1}(nz) = nf_\alpha(z) = 0$, and $f_{\alpha-1}$ is isomorphic. So we have $z \in T(H)$, and $f_1(z) = f_\alpha(z) = 0$; but f_1 is isomorphic, and so $z = 0$.

The desired extension f of f_1 is now given by $f(x) = f_1(x)$ for $x \in T(H)$ and $f(x_\alpha) = f_{\alpha+1}(x_\alpha)$ for the x_α which form the complement of $T(H)$.

§ 4. We come now to a theorem which is the "dual" of Theorem II.

THEOREM II*. *Let G be a compact group and let G_1 be its component of the identity; G is monothetic if and only if it is separable and the totally disconnected factor group G/G_1 is monothetic.*

For the proof we note that the (obviously necessary) separability guarantees that the power condition of Theorem II is fulfilled for $H = G^*$. Therefore, by Theorem II G^* is isomorphic to a subgroup of $\overline{C}$ if and only if $T(G^*)$ is. But $T(G^*)$ is, as is well known, the character group of G/G_1. Applying now Theorem I to G and G/G_1 we obtain Theorem II*.

From this follows immediately the

COROLLARY. *Every compact connected separable (abelian) group is monothetic.*

A discrete group is called *locally cyclic* if every subgroup which is generated by a finite number of elements can be generated by a single element. It is easy to see that a group without elements of infinite order is locally cyclic if and only if it is isomorphic to a subgroup of $T(\overline{C})$. We may accordingly restate Theorem II* as

THEOREM II'. *A compact group G is monothetic if and only if it is separable and the torsion group $T(G^*)$ of its character group is locally cyclic.*

Let Z be a compact totally disconnected monothetic group. Since the character group of a totally disconnected group has no elements of infinite order it follows from Theorem I that Z^* is (isomorphic to) a subgroup of $T(\overline{C})$. Consequently Z^* is the direct sum of groups Z^*_p, p running over the prime numbers, where each Z^*_p is either the zero group or cyclic of order p^n

for some n or isomorphic to the group T_p of all elements of $T(\overline{C})$ the order of which is a power of p. It follows that Z is the direct sum of groups Z_p, p running over the primes, where Z_p is either the zero group or cyclic of order p^n for some n or the p-adic group, the p-adic group being the character group of the group T_p just mentioned. Conversely, every such direct sum is a compact totally disconnected monothetic group.

§ 5. We prove now a theorem on the Haar measure of the set of generators of a group which is a partial strengthening of the corollary of § 4.

THEOREM III. *The set of generators of a compact connected (abelian) group satisfying the second countability axiom has Haar measure 1.*[3]

Proof. The character group G^* is countable; let $0, x_1, x_2, \ldots$ be its elements. Let G_i be the set of those elements of G which are mapped by the character x_i of G into the zero of C. Each G_i is a closed proper subgroup of G, and so of measure 0 (because, G being connected, G_i has an infinite number of disjoint cosets of equal measure); the union of the G_i has therefore measure 0 too. Using now remark (c) of §2 we see that the set of generators of G is identical with the complement of the union of the G_i, and so of measure 1.

Theorem III is not necessarily true for a group which does not fulfil the second countability axiom. An example is the toral group of dimension $\mathfrak{c}$ (the direct sum of $\mathfrak{c}$ copies of the group C); the set of its generators has inner measure 0 and outer measure 1. To see this, we call a subset of that group a c-set if it is (in an obvious sense) a cylinder set over a countable number of coördinates. It has been shown to us by S. Kakutani that the inner measure of a subset S of our group is equal to the supremum of the measures of measurable c-sets contained in S. Now it is clear that an element of the group is a generator if and only if its coördinates are linearly independent elements of $\overline{C}$. Therefore every c-set contains elements which are not generators; and this means that the inner measure of the set of generators is 0. On the other hand it is easily seen that every c-set of positive measure contains a generator (using Theorem III for the special case, $G =$ direct sum of countably many copies of C); hence the complement of the set of generators is of inner measure 0, or the outer measure of the set of generators is 1.

The situation for totally disconnected monothetic groups is this. The set of generators of a group which is cyclic of order p^n, or p-adic, has measure $1 - 1/p$. From this and the structure of an arbitrary totally disconnected monothetic group Z (§ 4) one concludes easily that the measure of the set of generators of Z is equal to $\Pi (1 - 1/p)$, extended over those primes p for which $Z_p \neq 0$, and hence that this measure may take any value between 0 and 1.

[1] Van Dantzig, D., "Zur topologischen Algebra," *Mathematische Annalen*, **107**, 591 (1933).

[2] Weil, André, "L'integration dans les groupes topologiques et ses applications," Paris, p. 97 (1938).

[3] This theorem was stated by Schreier, J., and Ulam, S., "Sur le nombre des générateurs d'un groupe topologique compact et connexe," *Fund. Math.*, **24**, 304 (1935).

ON THE DIRICHLET PROBLEM FOR THE HYPERBOLIC CASE

By J. Hadamard

New York City

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After having ascertained that Cauchy's problem admits of no solution in general, for equations of the elliptic type, it is natural to investigate whether, conversely, such is the case as concerns Dirichlet's problem for hyperbolic equations: to begin, for any hyperbolic equation in two independent variables

$$\frac{\partial^2 u}{\partial x \partial y} + A(x, y)\frac{\partial u}{\partial x} + B\frac{\partial u}{\partial y} + Cu = 0. \qquad \text{(E)}$$

The question is to determine a solution u of such an equation, being given the values of u along a closed contour C. It is assumed, for simplicity, that C is convex with respect to both characteristic directions, i.e., met at no more than two points by any parallel to the x- or the y-axis (unless it contains a segment of such a parallel) so that either x or y has only one maximum and one minimum on C. Points where such a maximum or minimum takes place will be called "vertices" of C, a "side" being the segment of C between two consecutives vertices.

I have dealt with that question from 1921 on.[1] It can be easily seen that such a Dirichlet problem admits of no solution when C has only two or three vertices, or, also, when it consists of a rectangle $MNPQ$ with its sides parallel to the axes. But in the case of four vertices—e.g., when C is an ellipse—the problem had hitherto been treated only for the simplest equation

$$\frac{\partial^2 u}{\partial x \partial y} = 0, \qquad \text{(e)}$$

where discussion has been carried out thanks to the fact that, for any solution of (e) and for any rectangle such as $MNPQ$, we must have

$$u_M + u_P = u_N + u_Q, \qquad (1)$$

this leading to the conclusion that the problem is, in general, again im-

possible, the conditions of possibility depending, in an unforeseen manner, on Diophantine properties of the contour.[2]

Such are the results as concerns (e). As we said, a great part of them is related to the identity (1), the analogue of which does not exist for other equations of the type (E), even for the simplest class of them after (e), i.e., equations which are integrated by an immediate application of Laplace's method. Attempts to generalize them to such equations remained unsuccessful until, recently, I have discovered that the conclusions are of a quite different nature.

1. The general solution of an equation which is integrated by an immediate application of Laplace's method can be, by means of an insignificant change of unknown, written[3]

$$u = X + \int \lambda Y dy, \tag{2}$$

where X denotes an arbitrary function of x; Y, an arbitrary function of y; $\lambda = \lambda(x, y)$, a determinate function of x and y, the equation itself being

$$\frac{\partial^2 u}{\partial x \partial y} - \frac{1}{\lambda}\frac{\partial \lambda}{\partial x}\frac{\partial u}{\partial y} = 0. \tag{e'}$$

λ must not vanish inside C if we want the coefficients of the equation to be regular.

We shall limit ourselves to contours C admitting the y-axis as an axis of symmetry, so that the two ordinates $\bar{y}$ and $\underline{y} < \bar{y}$ which correspond to the same value of x are even functions of x, the maximum of $\bar{y}$ and the minimum of $\underline{y}$ occurring for $x = 0$.

If λ simply consists of a product $f(x)g(y)$, the equation is reducible to (e) by changing u into $u/f(x)$. We begin by taking the next case, in which λ is a sum of two such terms, which case may generally be reconducted to assuming $\lambda = x + \varphi(y)$.

Now, we are given, for every value of x between $-a$ and $+a$, not only two values y and $\underline{y}$ of y, but two corresponding values $\bar{u}$ and $\underline{u}$ of u and we must have

$$X + \int_0^{\bar{y}} \lambda Y dy = \bar{u}, \tag{3}$$

$$X + \int_0^{\underline{y}} \lambda Y dy = \underline{u}. \tag{3'}$$

These, by subtracting and writing (u) for $\bar{u} - \underline{u}$,

$$\int_{\underline{y}}^{\bar{y}} \lambda Y dy = (u). \tag{4}$$

Conversely, it will be sufficient to satisfy the latter condition: for, then, we can choose X by (3) and it will satisfy (3').

Moreover, we shall consider simultaneously opposite values x and $-x$ of the abscissa and combine the corresponding equations (4) by addition and subtraction. If we write, for every x between 0 and a

$$\begin{aligned}(u)(x) + (u)(-x) &= 2v(x),\\ (u)(x) - (u)(-x) &= 2xw(x),\end{aligned} \tag{5}$$

we shall have, for $\lambda = x + \varphi(y)$,

$$\int_{\underline{y}}^{\overline{y}} Y dy = w(x), \qquad \int_{\underline{y}}^{\overline{y}} \varphi(y) Y dy = v(x).$$

These equations are both satisfied for $x = a$, each member being zero, so that we can replace them by their derivatives with respect to x, viz.,

$$\overline{y}'\overline{Y} - \underline{y}'\underline{Y} = dw/dx, \quad \varphi(\overline{y})\overline{y}'\overline{Y} - \varphi(\underline{y})\underline{y}'\underline{Y} = dv/dx \tag{6}$$

$\overline{Y}$ and $\underline{Y}$ standing for $Y(\overline{y})$, $Y(\underline{y})$; or, solving with respect to $\overline{Y}$, $\underline{Y}$,

$$\overline{Y} = \frac{dv/d\overline{y} - \varphi(\underline{y})dw/d\overline{y}}{\overline{\varphi} - \underline{\varphi}} \tag{6'}$$

with a similar formula for $\underline{Y}$. If we assume φ to be a monotone function of y (or even under more general circumstances), the denominator will not vanish except as the vertices $x = \pm a$. If φ_0 denotes the value of φ at one of these vertices, we have a condition of possibility, viz.,

$$dv/dy - \varphi_0 dw/dy = 0, \tag{7}$$

which is common to (6') and the formula for $\underline{Y}$, at the aforesaid vertices. This will be sufficient in order that $\overline{Y}$ and $\underline{Y}$ exist, if (1) at such a vertex, φ has a derivative different from zero; (2) the left-hand member of (7) admits of a derivative. But a second condition of possibility is required if we want $\overline{Y}$ and $\underline{Y}$ to be equal (in other words, Y not be affected with a discontinuity of the first kind).

At the vertices on the y-axis, $\overline{Y}$ and $\underline{Y}$ will, on the contrary, become infinite (excepting, however, the hypothesis of angular points at those vertices) because such is the case for $dx/d\overline{y}$ or $dx/d\underline{y}$; but $\int \frac{dx}{d\overline{y}} d\overline{y}$ and $\int \frac{dx}{d\underline{y}} d\underline{y}$ being absolutely convergent, this will not prevent u from being continuous around these points.

Such a result is of an unexpected simplicity, implying one single condition or two conditions of possibility, in contrast with the (in general, transcendental) system of conditions which intervenes when we deal with equation (e).

2. For arbitrary forms of λ, the ordinary equations (6) will be replaced

by integral equations of the Volterra type. To simplify scripture, we shall now assume C to be symmetrical with respect not only to the y-, but also to the x-axis, so that $\underline{y} = -\overline{y}$: a, strictly speaking, unnecessary assumption, but which does not diminish generality, as is easily seen by means of a transformation carried out on the coördinate y. Thus (4) can be written

$$\int_0^{\overline{y}} [\lambda(x, y)\overline{Y} + \lambda(x, -y)\underline{Y}]dy = (u),$$

now implying two unknown functions $\overline{Y}$, $\underline{Y}$ in the interval $(0, b)$, where b is the maximum ordinate on C. As above, we write it for opposite values of x and combine by addition and subtraction, getting

$$\int_0^{\overline{y}} [\beta(x, y)\overline{Y} + \beta(x, -y)\underline{Y}]dy = v, \quad \int_0^{\overline{y}} [\gamma(x, y)\overline{Y} + \gamma(x, -y)\underline{Y}]dy = w$$

where

$$\lambda(x, y) + \lambda(-x, y) = \beta(x, y), \quad \lambda(x, y) - \lambda(-x, y) = 2x\gamma(x, y),$$

while v, w still keep their meanings (5). These are two Volterra equations of the first kind, the integrands depending on y by the intermediary of x. As classical, we reconduct them to the second kind by differentiation (the equations being evidently satisfied for $y = 0$) and we get

$$\overline{\beta}\overline{Y} + \underline{\beta}\underline{Y} + \frac{dx}{d\overline{y}} \int_0^{\overline{y}} \left[\frac{\partial \beta}{\partial x}(x, y)\overline{Y} + \frac{\partial \beta}{\partial x}(x, -y)\underline{Y}\right] dy = \frac{dv}{d\overline{y}},$$

$$\overline{\gamma}\overline{Y} + \underline{\gamma}\underline{Y} + \frac{dx}{d\overline{y}} \int_0^{\overline{y}} \left[\frac{\partial \gamma}{\partial x}(x, y)\overline{Y} + \frac{\partial \gamma}{\partial x}(x, -y)\underline{Y}\right] dy = \frac{dw}{d\overline{y}}$$

$\overline{\beta}$, $\underline{\beta}$, $\overline{\gamma}$, $\underline{\gamma}$ standing for $\beta(x, \overline{y})$, $\beta(x, -\overline{y})$, $\gamma(x, \overline{y})$, $\gamma(x, -\overline{y})$.

We again assume that the determinant

$$D = \overline{\beta}\underline{\gamma} - \underline{\beta}\overline{\gamma}$$

is different from zero except at the vertices (for instance, that β/γ is a monotone function of y along the contour). At the vertices on the x-axis, D vanishes necessarily and we have the condition of possibility

$$\gamma_0 \frac{dv}{d\overline{y}} - \beta_0 \frac{dw}{d\overline{y}} = 0 \qquad (7')$$

which is the analogue of (7), β_0 and γ_0 being the values of β and γ for $x = a$, $y = 0$. This will, conversely, prove sufficient (except for the continuity of $\overline{Y}$ and $\underline{Y}$), if D is only of the first order in y (that is, if $\beta \frac{d\gamma}{dy} - \gamma \frac{d\beta}{dy} \neq 0$ at the vertex) and if, moreover, the left-hand member of (7') has a derivative: for $dx/d\overline{y}$ is, as we can assume, at least of the order of y in the

vicinity of $y = 0$ and, therefore, the product $D^{-1}\frac{dx}{d\bar{y}}$ will remain regular for $y = 0$, so that no singularity will occur except, generally, a discontinuity of the first kind. For any positive $\bar{y}$ less than b, such equations will be solved by the classical formulae of Volterra. For $y = b$, there will be again no difficulty if the corresponding vertex is an angular point. If there is a tangent parallel to the x-axis, $dx/d\bar{y}$ will become infinite[4] and the same takes place, as a rule, for the solutions $\overline{Y}$, $\underline{Y}$, as already appears on the special case treated in the preceding section; but, if we introduce the function

$$\xi(\bar{y}) = \frac{1}{\bar{y}}\frac{dx}{d\bar{y}}$$

and the new unknowns $\overline{Z}$, $\underline{Z}$ defined by

$$\overline{Y}(\bar{y}) = \xi(\bar{y})\overline{Z}(\bar{y}), \quad \underline{Y}(\bar{y}) = \xi(\bar{y})\underline{Z}(\bar{y}),$$

we shall now see that $\overline{Z}$, $\underline{Z}$ will remain finite, so that, as in the preceding section, u will remain continuous. The equations for $\overline{Z}$, $\underline{Z}$ will be of the form

$$\left.\begin{aligned}\overline{Z}(\bar{y}) + \int_0^{\bar{y}} \xi(y)[H\overline{Z}(y) + J\underline{Z}(y)]dy &= \frac{\bar{y}}{D}\left(\gamma\frac{dv}{dx} - \beta\frac{dw}{dx}\right)\\ \underline{Z}(\bar{y}) + \int_0^{\bar{y}} \xi(y)[K\overline{Z}(y) + L\underline{Z}(y)]dy &= \frac{\bar{y}}{D}\left(\beta\frac{dw}{dx} - \gamma\frac{dv}{dx}\right)\end{aligned}\right\}, \quad (8)$$

(H, J, K, L, finite functions of y, $\bar{y}$). For any shape of C in the vicinity of the vertex (under our general assumptions) $\xi(y)$ will be such that the integral $\int \xi(y)dy$ converges. Therefore, in the Volterra series which solve (8), each of the successive terms

$$\left.\begin{aligned}\overline{Z}_h &= -\int_0^{\bar{y}} \xi(y)[H\overline{Z}_{h-1}(y) + J\underline{Z}_{h-1}(y)]dy\\ \underline{Z}_h &= -\int_0^{\bar{y}} \xi(y)[K\overline{Z}_{h-1}(y) + L\underline{Z}_{h-1}(y)]dy\end{aligned}\right\} \quad (9)$$

will remain finite even for $\bar{y} = b$. Let us write

$$\overline{Z}_h = \overline{A}_h\bar{y}^h, \quad \underline{Z}_h = \underline{A}_h\bar{y}^h, \quad |\overline{A}_h|, |\underline{A}_h| \leqslant M_h.$$

If $\xi(y)$ remains finite, it is elementary that M_h/M_{h-1} would tend to zero like $1/h$. But at any rate, we have

$$M_h\bar{y}^h \leqslant kM_{h-1}\int^1 \xi(y)y^{h-1}dy \quad (k, \text{ a positive constant})$$

Let us assume that, for any y, y_1 such that $0 < y_1 < y$, the ratio $\xi(y_1)/\xi(y)$ remains less than a fixed number l: this is certainly true if C is con-

vex in the ordinary meaning of the word (so that $|dx/dy|$ is an increasing function of y) and x is of the second order for $y = 0$. Let us also, by a change of units, take $b = 1$. The integral

$$I_h = \int_0^1 \xi(y) y^{h-1} dy$$

tends to zero when h increases to infinity. Then, the analogous integral with the upper limit $\bar{y}$ can be written

$$\int_0^{\bar{y}} \xi(y) y^{h-1} dy = \bar{y}^h \int_0^1 \xi(t\bar{y}) t^{h-1} dt \leqslant \bar{y}^h I_h \cdot l,$$

on account of the assumption concerning $\xi(y)$. Therefore, we can take

$$M_h \leqslant klM_{h-1} I_h,$$

where the last factor tends to zero,[5] and we see that the series for $\bar{Z}$, $\underline{Z}$ are uniformly convergent for every y between 0 and 1, limits included.

3. The results are, as we see, mainly the same as in the preceding section. But they are in full discrepancy with what has been found for (e)—and also with what happens for any equation of the type (E) whenever the contour has only two or three vertices—as only one or two conditions of possibility are required, at the vertices $y = 0$, $x = \pm a$. Which of these so different conclusions gives us a right idea of what occurs in the general case? Perhaps neither of them: Poincaré has taught us to diffide of conclusions drawn, as to a general problem, from the special cases the treatment of which happens to be within our reach.

[1] *Proc. Benares Math. Soc.*, **3**, 39 (1921); *Lectures on Cauchy's Problem*, French edition, Paris, 1932, Appendix II; "Conferences Internationales des Sciences Math., Geneve, 1935," published in *L'Enseignement Mathematique*, Paris-Geneve, 1936; *Jour. Chinese Math. Soc.*, **2**, 6–20 (1937).

[2] Besides the papers mentioned in reference 1, above, see Huber, A., *Monatshefte Math. Phys.*, **39** (1932); Bourgin, D. G., and Duffin, R., *Bull. Am. Math. Soc.*, **45** (1939); and a beautiful treatment by John, F., *Am. Jourl.*, **63** (1941).

[3] The general solution of an equation admitting immediate integration by Laplace's method is written in Darboux's *Leçons*, vol. 2,

$$\alpha(X + \int \beta Y dy).$$

Both α and β being necessarily different from zero inside C, we do not diminish generality by taking $\alpha = 1$, and we have written λ instead of β.

[4] Such will be also the case for the right-hand members of the equations which will similarly become infinite like ξ. Near $(0, b)$, it is natural to assume v, w to be regular in x, but not, in general, in y.

[5] If, in the vicinity of the vertex on the y-axis, $1 - \bar{y}$ is of the order of x^m, with m being any constant greater than one, I_h will be $1/m\ B(h, 1/m)$. B, an eulerian integral of the first kind, is asymptotically equal to $\Gamma 1/m\ h^{-1/m}$, so that the series (9) will converge like $\Sigma(k'y)^h : (h!)^{1/m}$.